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# An Evaluation of the Groundwater Microbial Community's Toluene Biogenesis Potential at a Site in Southeast Louisiana

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# **AN EVALUATION OF THE GROUNDWATER MICROBIAL COMMUNITY'S TOLUENE BIOGENESIS POTENTIAL AT A SITE IN SOUTHEAST LOUISIANA**

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements of the degree of  
Master of Science in Civil Engineering

in

The Department of Civil and Environmental Engineering

by

Michael Aaron Griffin Jr.  
B.S, Louisiana State University, 2015  
May 2018

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## ABSTRACT

The broad goal of the research described in this thesis was to better understand the potential for biological toluene production to occur in groundwater at a Superfund Site located in southeast Louisiana. Previous literature reported that bacterial isolates under laboratory test conditions and undefined microbial communities in anaerobic sludge digesters and seasonally stratified lakes can produce toluene biogenically. Most of the research reported on this subject previously, however, has been on systems in Europe and little research has been reported previously on such phenomena in North America.

In the research described here, studies were conducted utilizing groundwater from a Superfund site employing an enhanced bioremediation strategy to reductively dehalogenate chlorinated solvents. When incubated at ambient room temperature ( $\sim 22^{\circ}\text{C}$ ) and provided glucose and either phenylalanine or phenylacetic acid, microbial communities originating from the groundwater displayed the ability to biogenically produce toluene. When provided with 0.8 mM (132 mg/L) phenylalanine, cultures grown in liquid media accumulated toluene to aqueous-phase concentrations 0.44 mM to 0.54 mM of toluene, 41 mg/L and 50 mg/L respectively, in less than 30 days. In experiments in which an enrichment culture derived from site groundwater was supplied with either phenylacetic acid or phenylalanine at concentrations ranging from 0-400 mg/L (0-2.42 mM and 0-2.94 mM respectively), there was a high correlation between toluene accumulated at the end of a 28-day incubation period and the concentration of phenyl-containing precursor supplied. The observed toluene yield was 0.52 moles toluene produced per mole phenylacetic acid supplied and 0.575 moles toluene produced per mole phenylalanine supplied. The toluene-producing capabilities of the enrichment cultures were maintained during propagation through four sequential transfers and after long-term ( $\sim 15$  months) storage at  $4^{\circ}\text{C}$ .

The finding that groundwater microbial communities in areas undergoing enhanced bioremediation to treat chlorinated solvent contamination can produce toluene at levels in excess of drinking water standards has important implications for the field of bioremediation. Further research is necessary to identify how widespread biogenic toluene production is in areas undergoing enhanced remediation. Further research is also required to identify the microbes responsible for toluene production.

# **1 INTRODUCTION**

Because it is a constituent of gasoline and other consumer products that have been widely released into the environment by human activities, the presence of toluene in many environments has generally been viewed as directly resulting from anthropogenic pollution. A growing body of research, however, suggests that a portion of the toluene found in the environment may not result from direct anthropogenic release of the compound, especially in cases where toluene is not accompanied by other BTEX (benzene/toluene/ethylbenzene/xylene) compounds.

During an effort that involved subsurface injection of agricultural feed grade molasses into the subsurface in an effort to stimulate indigenous bacterial populations' biodegradation of chlorinated aliphatic compounds at a Superfund site in south Louisiana, toluene was observed in the groundwater at concentrations that exceed drinking water standards.

Previous reports in the scientific literature demonstrated that toluene can be biologically produced from aromatic precursor compounds under anaerobic conditions. The literature reported finding of biological toluene production under natural conditions, such as seasonally stratified lakes, as well as more artificial conditions, namely anaerobic wastewater-sludge digesters. The thesis research described here describes an effort conducted to ascertain if a contaminated waste site supplemented with an easily fermentable substrate could similarly stimulate anaerobic microbial communities into biologically producing toluene at a faster rate than it could be degraded, resulting in toluene accumulation as an aqueous-phase pollutant.

## **1.1 Objectives and Approach**

The overall objective of the research described in this thesis was to better understand the potential for biological toluene production to occur in groundwater at a Superfund Site located in

southeast Louisiana. To achieve this objective, the research was divided into three tasks that involved:

- (1) Evaluation of the groundwater microbial populations' ability to produce toluene under laboratory conditions
- (2) Establishment and propagation of enrichment cultures, and
- (3) Testing to assess the ability of enrichment cultures to utilize various carbon sources and potential precursors for toluene production

## **1.2 Thesis Organization**

Chapter 2 of this thesis contains a literature review that contains a brief overview of toluene's properties, a discussion of what has been reported previously regarding biogenically produced toluene, and a description of bacteria currently classified in the genus *Tolomonas* with a particular emphasis on the species *Tolomonas auensis*. Chapter 3 describes concentrations of aromatic hydrocarbons detected at a Louisiana field site following the initiation of an *in situ* biostimulation remediation strategy as well as methods and results from a series of experiments performed to assess the capacity of the site's groundwater microbial community to produce toluene under laboratory test conditions. Chapter 4 contains overall conclusions and recommendations for future research. Chapter 5 contains a compilation of references cited throughout the thesis.



## 2 LITERATURE REVIEW

This chapter contains a review of literature related to multiple aspects of the thesis research. Section 2.1 provides an overview of toluene's industrial use, potential health hazards, and environmental regulations. Section 2.2 discusses previous research related to the potential for toluene biogenesis in the environment and in engineered waste treatment systems. Section 2.3 contains a detailed description of the genus *Tolomonus* and previous research regarding its potential to produce toluene. Section 2.4 describes previous research and anaerobic toluene biodegradation.

### 2.1 Overview of Toluene's Industrial Use, Health Hazards, and Environmental Regulations

Toluene ( $C_7H_8$ ) is used in a wide variety of industrial applications, primarily as a chemical feedstock in the production of benzene and xylene (ASTDR, 2015). It is also used to boost the octane rating in gasoline and is present in many consumer products such as paints, adhesives, resins and rubbers (ASTDR, 2015). Through accidental leaks and spills of various toluene-containing liquid fuels, and atmospheric emission of incompletely combusted materials, significant amounts of toluene have been introduced into the environment. In 2016, 21 million pounds (9.6 million kilograms) in toluene releases were reported in the US EPA Toxic Release Inventory (TRI), predominantly as point-source and fugitive air emissions (TRI, 2016). However, 45,000 pounds of toluene were reported as surface impoundments and land disposal, and 7,500 pounds of toluene were in surface water discharges reported (TRI, 2016). Because not all industrial facilities in the United States are required to report their releases to the TRI, the figures reported here serve as a lower-bound estimate of actual releases of toluene nationwide (ASTDR, 2015).

Long-term consumption of toluene-contaminated water has been linked to damage of the nervous system, kidneys, and liver (EPA, 2015). To protect public health, the United States Environmental Protection Agency (EPA) has set a maximum contaminant level (MCL) of 1 mg/L for toluene in drinking water (EPA, 2015). Inhalation of toluene-contaminated air also poses a health hazard. Locally high concentrations of gas-phase toluene may occur in indoor or outdoor air due to volatilization from household products containing toluene, cigarette smoke, and automobile exhaust (EPA 2015). The Occupational Safety and Health Administration (OSHA) regulatory permissible exposure limit (PEL) for toluene is set at 754 mg/m<sup>3</sup> (EPA 2015). Various physicochemical properties of toluene are summarized in Table 2.1.

**Table 2.1.** Physicochemical properties of toluene

Parameter	Value	Reference
Molecular Formula	C <sub>6</sub> H <sub>5</sub> CH <sub>3</sub>	pubchem.gov
CAS #	108-88-3	pubchem.gov
Molecular Weight (g/mole)	92.141	pubchem.gov
Density at 20°C (g/mL)	0.867	pubchem.gov
Boiling point (°C)	110.6	pubchem.gov
Log K <sub>ow</sub> (dimensionless)	2.73	pubchem.gov
Water Solubility at 25°C (mg/L)	526	pubchem.gov
Henry's Law Constant at 22°C (atm·m <sup>3</sup> /mol)	5.06×10 <sup>-3</sup>	Peng and Wan, 1997
(unitless)	0.209	
Vapor Pressure (kPa at 25 °C)	3.8	pubchem.gov

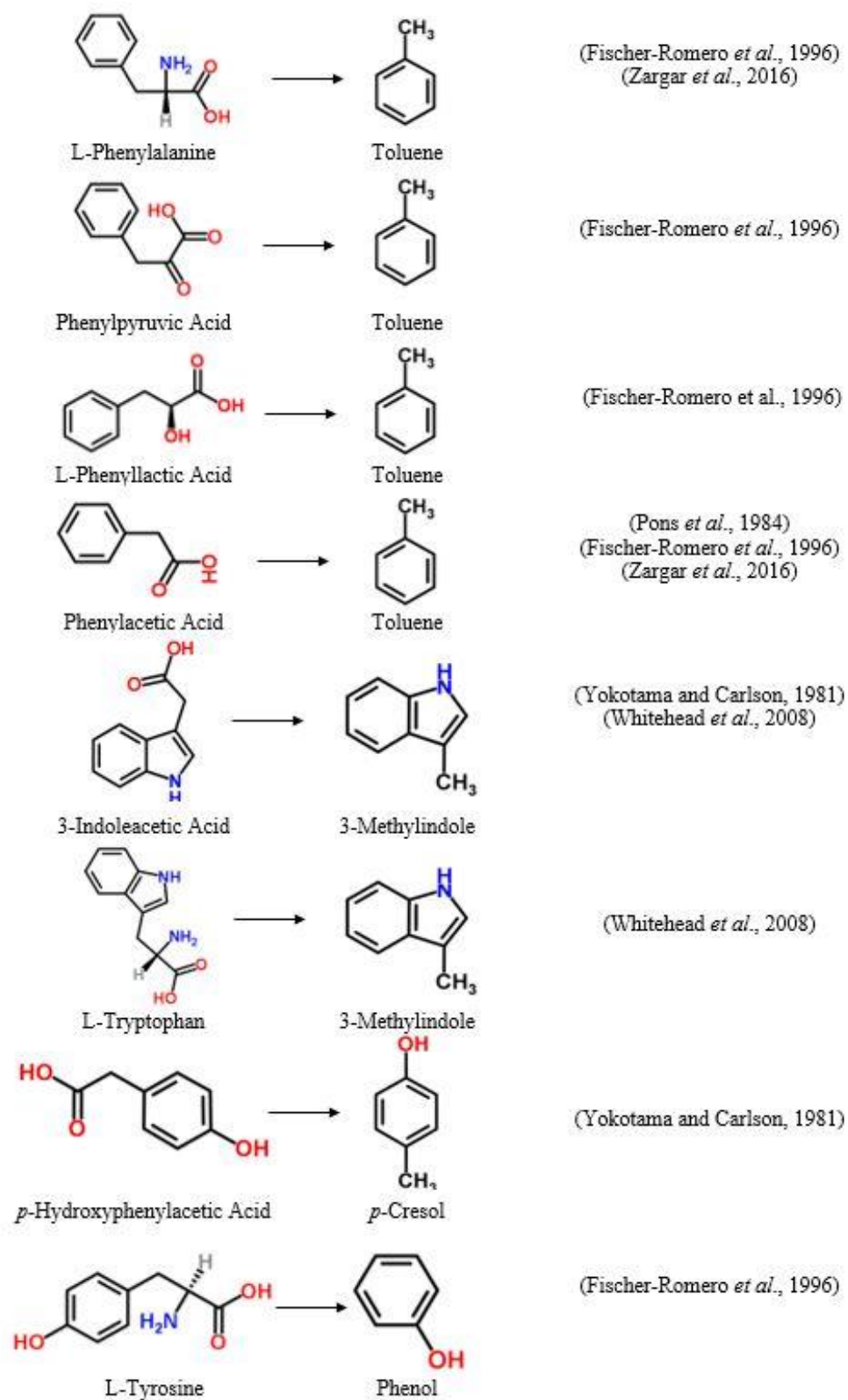
## 2.2 Biological Toluene Production

The presence of toluene in most urban and human-impacted environments is generally thought to originate from anthropogenic sources (e.g., pollution from refined petroleum products in widespread use). In the 1980s, however, evidence began to mount suggesting that contemporary biological sources could be responsible for some occurrences of aromatic hydrocarbons in the environment. The bacterium “*Clostridium aerofetidum*” strain WS was reported to produce toluene in the presence of phenylacetic acid or in the presence of both phenylalanine and

methionine together (Pons *et al.*, 1984). Other aromatic compounds such as phenol, *p*-cresol and skatole have also been shown to be biogenically produced from anaerobic transformation of amino acids that contain aromatic side chains (phenylalanine, tryptophan, and tyrosine), modified amino acids, or organic acids that contain an aromatic-group (Yokotama and Carlson, 1981; Fischer-Romero *et al.*, 1996; Whitehead *et al.*, 2008). A diagram of these compounds and their respective precursors are displayed in Figure 2.1.

Likely due to its use in consumer products and automobile fuels, toluene is frequently present in urban stormwater runoff and in the influent of wastewater entering municipal wastewater treatment plants. A study of VOCs in storm water runoff found that toluene was the most frequently detected BTEX compound (Delzer *et al.*, 1996). In 16 major cities across the United States, 62 different VOCs were measured in 592 samples of storm water runoff taken over a 4 years period (1991-1995) (Delzer *et al.*, 1996). While concentrations ranged from detectable levels (0.2 µg/L) to as high as 6.6 µg/L, the median concentration was only 0.3 µg/L (Delzer *et al.*, 1996).

Studies at two municipal wastewater treatment plants in Chicago reported that of the 11 VOCs detected in influent wastewater, toluene had by far the highest concentration of 86 µg/L, whereas all remaining VOCs were at much lower concentrations (< 20 µg/L) (Namkung and Rittman, 1987). Models indicated that of the five compounds known to be biodegraded under aerobic conditions (toluene, benzene, chlorobenzene, ethylbenzene, and methylene chloride), ~92% of the removal could be attributed to biodegradation. Adsorption as a mechanism for removal was determined to be negligible by models and was further confirmed when laboratory scale systems determined that adsorption of toluene to solids comprised less than 1% of removal mechanisms (Namkung and Rittman, 1987).



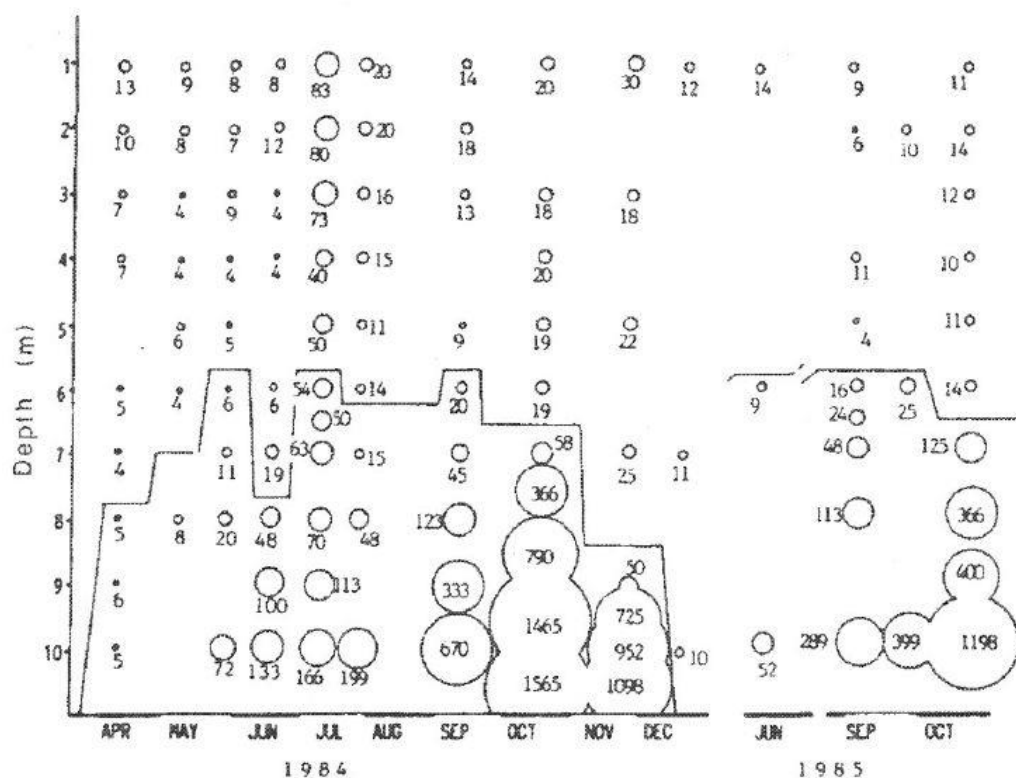
**Figure 2.1.** Chemical structures of compounds reported to be produced biogenically (right) and their respective precursors (left). [Structures taken from chemspider.com]

The presence of high concentrations of toluene in anaerobic sludge digesters of several municipal wastewater treatment plants in the United Kingdom were suspected to be of a biological source when concentrations of toluene exiting some treatment works were at slightly higher concentrations than that of the influent (Wilson *et al.*, 1994). Laboratory analysis of digester sludge samples taken from these treatment plants and were analyzed for 1,1-DCE; 1,2-DCE; 1,2-DCA; chloroform; 1,1-TCA; 1,1,2-TCA; TCE carbon tetrachloride, tetrachloroethene (PCE); benzene; toluene; ethylbenzene; *o*-xylene, and *m*- plus *p*-xylene (Wilson *et al.*, 1994). Of these compounds, toluene was by far the highest in concentrations among all volatile organic compounds (VOCs) analyzed, accounting for 91% of total mean aromatic VOC concentration (Wilson *et al.*, 1994). Analysis of sludge taken from 12 different wastewater treatment plants had a mean toluene concentration of  $87 \pm 172$  mg/kg (dry weight), with concentrations at one site of 549 mg/kg (14,900  $\mu\text{g/L}$ ) (Wilson *et al.*, 1994). While toluene concentrations in sludge varied greatly from site to site, no apparent correlation was found between the VOC concentrations found in wastewater treatment sludges and the percent of industrial influent, population served, solids content, or method of treatment used at each wastewater treatment plant (Wilson *et al.*, 1994).

Raw wastewater at one plant was reported to be as high as  $\sim 115$   $\mu\text{g/L}$ , while the effluent averaged 30  $\mu\text{g/L}$  (Suschka *et al.*, 1996). Field studies indicated that air stripping and aerobic biological mechanisms were estimated to account for 55.6% removal of toluene (Suschka *et al.*, 1996); however, when compared to measured concentrations in raw wastewater a toluene mass balance could not explain the high toluene concentrations measured in anaerobically digested sludge supernatant in comparison to measured concentrations of raw wastewater (Mrowiec *et al.*, 2005). This led to laboratory experiments of anaerobic sludge digestion, utilizing biosolids from one of the treatment plants studied, in Skoczow, Poland. While not identifying a specific organism

or pathway, it was noted that a strong correlation existed between increases in toluene concentrations during a time of increased volatile fatty acids during the anaerobic sludge digestion process (Mrowiec *et al.*, 2005). Their findings concluded, “biosynthesis of toluene occurs in the acidogenic phase [ORP -170 to -200 mV], while biodegradation was prevalent in the methanogenic phase [ORP -220 to -280 mv]” (Mrowiec *et al.*, 2005). Depending on the type of experiment, toluene levels in the first stage of anaerobic digestion increased from a base value of 200 µg/L to 20,000 and 42,000 µg/L (Mrowiec *et al.*, 2005). Under the subsequent methane-production stage of digestion, toluene was estimated to decrease at a rate ranging from 400 to 900 µg/L-day (Mrowiec *et al.*, 2005). Their finding “excluded the origin of toluene from external sources including the municipal wastewater itself” (Mrowiec *et al.*, 2005).

A substantial body of research has also been dedicated to investigating the presence of toluene in seasonally stratified lakes in Central Europe (Jüttner and Henatsch, 1986). Toluene concentrations fluctuated with seasons as well as depth, 0.008 µg/L at 1m depth and 0.133 µg/L at 10 m depth in June compared to 0.03 µg/L at 1 m and 1.565 µg/L at 10 m depth in October (see Figure 2.2); however anthropogenic compounds such as xylenes and ethyltoluenes were not detected (Jüttner and Henatsch, 1986). Similar results in six additional lakes that experienced anoxic stratification suggested that the findings are perhaps a widespread phenomenon (Jüttner, 1990). As the concentrations of toluene were found to be inversely related to concentrations of nitrate-N, they concluded that the formation of toluene, a likely result of anoxic degradation of protein matter, could surpass its degradation rate under anoxic denitrifying conditions (Jüttner, 1991).



**Figure 2.2.** Seasonal depth profiles of the concentrations of toluene (area of circles). The numbers beside or within the circles represent the toluene concentrations (ng l<sup>-1</sup>). The stepped horizontal line indicates the upper boundary of the anoxic part in the Schleinsee 1984 and 1985. [Reprinted by permission from Macmillan Publishers Ltd: Nature, Jüttner F. and Henatsch J.J., Anoxic Hypolimnion is a Significant Source of Biogenic Toluene. 323: 797 – 798. © 1986]

In addition, to further investigate a potential for biogenic toluene production in freshwater lake systems, samples of anoxic freshwater sediments collected from Lake Edebergsee (Holstein, North Germany) were used to inoculate basal medium under laboratory conditions. Utilizing glucose as a carbon source and various phenyl-containing precursors, at a concentration of 0.4 mmol/L, toluene was confirmed to be biogenically produced, using glucose as a carbon source, from: phenylacetate, phenylpyruvate, phenyllactate, and phenylalanine (Jüttner, 1991). The chemical structures for these compounds can be seen in Figure 2.1. Jüttner (1991) also noted that unlike the previous finding of Pons *et al.* (1984), when utilizing phenylalanine, the presence of methionine had an inhibitory effect on toluene production in their laboratory culture(s) inoculated with lake sediment (Jüttner, 1991). Samples which used phenylalanine as a precursor had the

shortest initial lag period of approximately 7 days before toluene production was recorded, and then showed a ten-fold increase in toluene concentrations within 12 days of inoculation (Jüttner, 1991). However, phenylacetate had the highest formation of toluene, accumulating to 25.2 mg/L, after 31 days of incubation (Jüttner, 1991). Results of various phenyl precursors are shown in Table 2.2. When the undefined mixed culture was supplied with radiolabeled phenylalanine [ $L$ -phenyl- $d_5$ -alanine-3,3- $d_2$  (99.4 atom % D)], radiolabeled toluene was produced and after a 27-day incubation unlabeled toluene made up less than 1% of total toluene produced by the undefined mixed culture (Jüttner, 1991).

**Table 2.2.** The effect of different precursor compounds (0.4 mmol/l) on the formation of toluene after 31 days incubation. Reprinted from: Springer, Verh Int Verein Limnol, Jüttner F., (1990) Distribution of Toluene in Stratified Lakes and River Dams of Southwest Germany. 24: 279 – 281. With permission of Springer.

Precursor	Toluene (mg/L)
Phenylacetate	25.2
Phenylpyruvate	19.0
Phenylalanine	13.0
Phenyllactate	12.3
2-Phenylethanol	5.3
Phenylacetaldehyde	1.7
Ethyl-phenylacetate	1.4
Benzaldehyde	0.05
Cennamate	0.04
Null	0.001

### 2.3 Detailed Description of the Genus *Tolomonus*

An important step in understanding potential biogenic toluene production came in 1996 when Fischer-Romero *et al.* (1996) reported the isolation and taxonomic characterization of a toluene-producing bacterium originating from anoxic sediments collected from Lake Au, a separate part of Lake Zurich, Switzerland. Strain TA 4<sup>T</sup> was capable of both growth and toluene production when transferred to liquid medium (Fischer-Romero *et al.*, 1996). This nonmotile, Gram-negative rod shaped (0.9 to 1.2 by 2.5 to 3.2  $\mu$ m) bacterium produced toluene using



phenylalanine, phenyllactate, phenylpyruvate and phenylacetate and a carbon source and was designated as the type strain of a new genus and species with name *Tolumonas auensis* (Fischer-Romero *et al.*, 1996). Type strain TA 4<sup>T</sup> was also reported to transform tyrosine into phenol (Fischer-Romero *et al.*, 1996). Toluene formation, which only occurred when one of these precursors and a carbon source were present, by strain TA 4<sup>T</sup> was reported at a temperature range of 12° - 25° C, and at pH ranging from 6.0 to 7.5, with optimum production at 22° C and 7.2 respectively (Fischer-Romero *et al.*, 1996). Strain TA 4<sup>T</sup> reportedly grew anaerobically on a wide variety of sugars tested, including D-arabinose, D-cellobiose, D-fructose, D-glucose, glycogen, Inulin, maltose, D-mannose, D-melezitose, melibiose, D-raffinose, L-rhamnose, D-ribose, salicin, sucrose, and D-trehalose (Fischer-Romero *et al.*, 1996). It did not grow anaerobically on cellulose, L-fucose, D-lactose, or D-xylose (Fischer-Romero *et al.*, 1996). Major fermentation products were acetate, formate, and ethanol when grown on glucose. Both growth and toluene production were reportedly observed under aerobic conditions as well (Fischer-Romero *et al.*, 1996).

The type strain of a second novel bacteria species in the genus *Tolumonas*, *Tolumonas osonensis*, was first isolated from anoxic freshwater sediment from Oso Creek in Corpus Christie, Texas (Caldwell *et al.*, 2011). The type strain, designated as OCF 7<sup>T</sup>, is a Gram-negative, non-motile, facultatively anaerobic bacterium grew optimally at 22° C and pH 7.5 (Caldwell *et al.*, 2011). Major fermentation products from glucose were formate, acetate, ethanol and lactate. Comparative 16S rRNA gene sequence analysis indicated that strain OCF 7<sup>T</sup> was phylogenetically related to the type strain of *Tolumonas auensis* (97.2% similarity) (Caldwell *et al.*, 2011). While strain OCF 7<sup>T</sup> did not exhibit the ability to transform phenylacetate, phenylalanine and substituted aromatic acids into toluene, biochemical and polar lipid data are all compatible with the genus *Tolumonas* (Caldwell *et al.*, 2011). Caldwell *et al.* (2011) thus emended the *Tolumonas* genus

description with the following changes: “May or may not produce toluene from phenylacetate or phenylalanine in the presence of another carbon source. May or may not produce phenol from tyrosine in the presence of another carbon source. May or may not utilize arabinose, fumarate, lactose or pyruvate. May or may not have the ability to hydrolyse urea. End products from glucose are acetate, ethanol, formate and/or lactate. Grows between pH 5.5 and 8.5.” (Caldwell *et al.*, 2011).

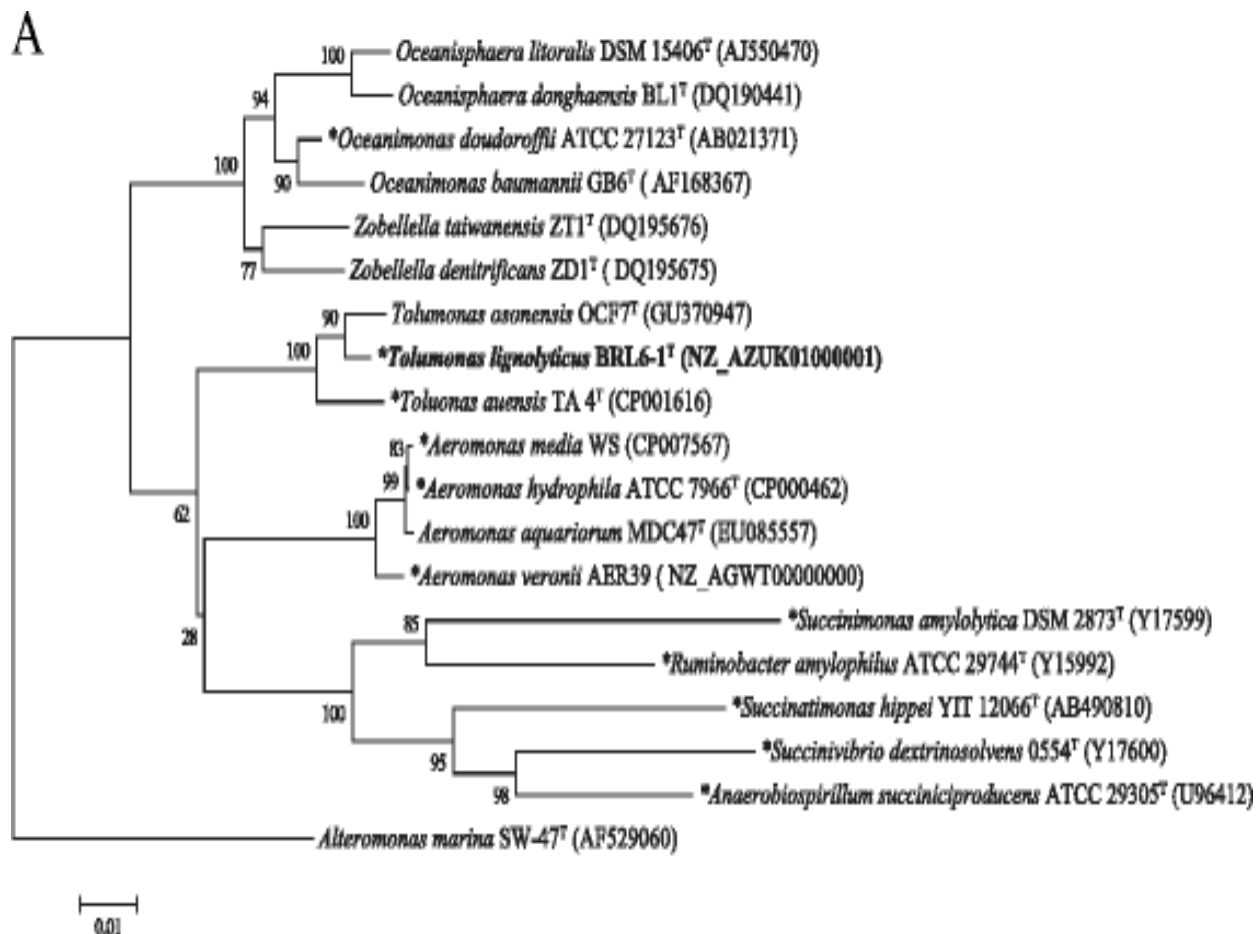
In 2015, a third novel species in the genus *Tolumonas*, *T. lignolytica*, was reported following isolation from rain forest soil samples collected from Puerto Rico (Billings *et al.*, 2015). Cells of BRL6-1<sup>T</sup>, the type strain of *T. lignolytica*, was described as mesophilic, non-spore forming, Gram-negative rods that are oxidase and catalase negative and had been isolated based on its ability to utilize lignin as a sole carbon source (Billings *et al.*, 2015). The strain grew optimally at a temperature of 30° C and a pH of 7. Growth was noted to occur from 15-37° C, and grew well aerobically and anaerobically (Billings *et al.*, 2015). Carbon sources which supported anaerobic growth included N-acetyl-D-glucosamine, L-arabinose, D-fructose, D-galactose, D-gluconic acid, α-D-glucose, L-lyxose, maltose, D-mannitol, D-mannose, matotriose, D-melibiose, D-robiose, D-sorbitol, sucrose, Tween 20, Tween 40, Tween 80, and D-trehalose (Billings *et al.*, 2015). A summary comparison between the species of the genus *Tolumonas* is shown in Table 2.3, and the placement of type strains from the genus *Tolumonas* relative to other bacteria in the order *Aeromonadales* in a phylogenetic tree constructed based on 16S rRNA gene sequences is shown in Figure 2.3.

**Table 2.3.** Comparison of various properties of the type strains of the three species currently assigned to the genus *Tolumonas*.

Species name	<i>T. auensis</i>	<i>T. osonensis</i>	<i>T. lignolytica</i>
Type strain	DSM 9187 <sup>T</sup>	OCF 7 <sup>T</sup>	BRL6-1 <sup>T</sup>
Temp range (optimum)	12 – 25°C (22°C)	15 – 37°C (22°C)	15-37°C (30°C)
pH range (optimum)	6.0 – 7.5 (7.2)	5.5-8.5 (7.5)	4.5-8.5 (7)
Produces toluene	Yes	No	Not reported
Produces phenol	Yes	No	Not reported
Hydrolysis of Urea	No	Yes	Not reported
Major fermentation products from glucose	Acetate, ethanol, formate	Acetate, ethanol, formate, lactate.	Not reported
Motility	Non-motile	Non-motile	Not reported
Gram Test	Negative	Negative	Negative
DNA mol% G+C content	52%	49%	47.56%

While Fischer-Romero (1996) described the genus *Tolumonas* and the species *T. auensis* based on the ability of the type strain TA 4<sup>T</sup> to produce toluene, Zargar *et al.* (2016) reported that after obtaining the type strain (=DSM 9187<sup>T</sup>) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), they were unable to replicate the anaerobic or aerobic biosynthesis of toluene from phenylacetate toluene in media described by Fischer-Romero *et al.* (1996) or in DSMZ Medium 500. While unable to replicate toluene production by the type strain of *T. auensis*, Zargar *et al.* (2016) were successful in developing a toluene-producing enrichment culture using sludge from the East Bay Utility District wastewater treatment plant (Oakland, CA) as the inoculum in a growth medium modified from the TP Medium published by Fischer-Romero (Zargar *et al.*, 2016). The modification of the growth medium utilized by Zargar *et al.* (2016) included replacing the bicarbonate buffer with an organic buffer (HEPES, 18 mM) and reducing the sulfate concentration (in order to limit sulfide production) by replacing a portion of MgSO<sub>4</sub> with MgCl. A side-by-side comparison of the media formulations can be found in Table A1 of Appendix A. The recovery of <sup>13</sup>C labeled toluene from Zargar *et al.* (2016) enrichment cultures supplied with <sup>13</sup>C labeled phenylacetic acid and <sup>13</sup>C labeled phenylalanine provide compelling

evidence of toluene biosynthesis from these substrates even if the responsible microorganisms have not yet been identified.



**Figure 2.3.** Phylogenetic tree highlighting the position of *Tolumonas lignolytica* BRL6-1<sup>T</sup> among the *Aeromonadales*. The phylogenetic tree based on 16S ribosomal RNA gene sequence was inferred using the Neighbor-Joining method within MEGA6. Bootstrap values of 1000 replicate trees are shown at the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated, creating a total of 1234 positions in the final dataset. GenBank accession numbers are shown in parentheses after strain numbers. Type strains are indicated with a *superscript* T. Organisms with genomes available are indicated by and *asterisk* before the name. [Figure and caption re-printed from Billings *et al.*, 2015]

In experiments utilizing the mixed culture derived from wastewater sludge in conjunction with labeled <sup>13</sup>C-phenylacetic acid, an approximately 1 to 1 molar ratio of <sup>13</sup>C-toluene produced

per  $^{13}\text{C}$ -phenylacetate consumed was determined (Zargar *et al.*, 2016). For cultures supplied with  $^{13}\text{C}$ -phenylalanine, the molar ratio of  $^{13}\text{C}$ -toluene produced per  $^{13}\text{C}$ -phenylalanine consumed was 0.35 to 1 (Zargar *et al.*, 2016). Cell-free extracts provided with both phenylacetate and *p*-hydroxyphenylacetate showed a resulting correlation of toluene and *p*-cresol, suggesting that the same enzyme could be responsible for catalyzing decarboxylation of both phenylacetate and *p*-hydroxyphenylacetate (Zargar *et al.*, 2016). Furthermore, activities of cell-free extracts were irreversibly inactivated by exposure to oxygen (Zargar *et al.*, 2016). Using, fast protein liquid chromatography (FPLC) fractionation, notably absent from the proteins of the toluene-producing mixed culture was any identification of the well-characterized Clostridial *p*-hydroxyphenylacetate decarboxylase (CsdBC) protein (Zargar *et al.*, 2016). In addition, experimentation utilizing CsdBC showed it was unable to catalyze phenylacetate decarboxylation (Zargar *et al.*, 2016). This led Zargar *et al.* (2016) to conclude that toluene production was catalyzed by a novel phenylacetate decarboxylase rather than *p*-hydroxyphenylacetate decarboxylase (Zargar *et al.*, 2016).

## **2.4 Biological Toluene Degradation**

Prior to the 1980s it was widely believed that microbial degradation of toluene was not possible in the absence of oxygen (Grbic-Galic and Vogel, 1987). Since that time, however, a multitude of studies published in the literature on the topic of toluene biodegradation have demonstrated that toluene may be biodegraded under denitrifying, sulfate-reducing, dissimilatory iron-reducing, and methanogenic conditions. As further discussed below, several bacterial species with an ability to biodegrade toluene in the absence of oxygen have been reported.

Under sulfate-reducing conditions, supporting evidence has been shown that sulfate was the terminal electron acceptor during toluene degradation by mixed cultures (Edwards *et al.*, 1992). Several factors, including pH, availability of sulfate and/or preferred substrates, and concentrations

of both toluene and sulfide created great variability in the rates of toluene degradation. In several instances, cultures that produced higher concentrations of sulfide had reduced rates of toluene degradation in mixed cultures (Edwards *et al.*, 1992; Beller and Reinhard, 1995; Huang *et al.*, 2016) as well as isolate strains (Beller and Spormann 1997). Utilizing a mixed culture from gasoline contaminated silt, the optimum pH for anaerobic toluene degradation was near 7.0, while degradation rate was 20% less at 6.0 and 40% less at pH 8.0 (Edwards *et al.*, 1992). The accumulation of sulfide as a result of sulfate reduction also appeared to be inhibitory; as well, the addition of 1 mM NaS reduced toluene degradation rates by half (Edwards *et al.*, 1992). Furthermore, the presence of more easily degradable substrates, such as lactate, glucose and yeast extract caused toluene degradation to cease entirely until the preferred substrates had been consumed (Edwards *et al.*, 1992). Toluene degradation also ceased when sulfates became depleted, and resumed upon addition of sulfate (Edwards *et al.*, 1992). Overall, the rates of toluene degradation observed in this mixed culture, ranged from 0.1 to 1.5 mg/L/day (Edwards *et al.*, 1992).

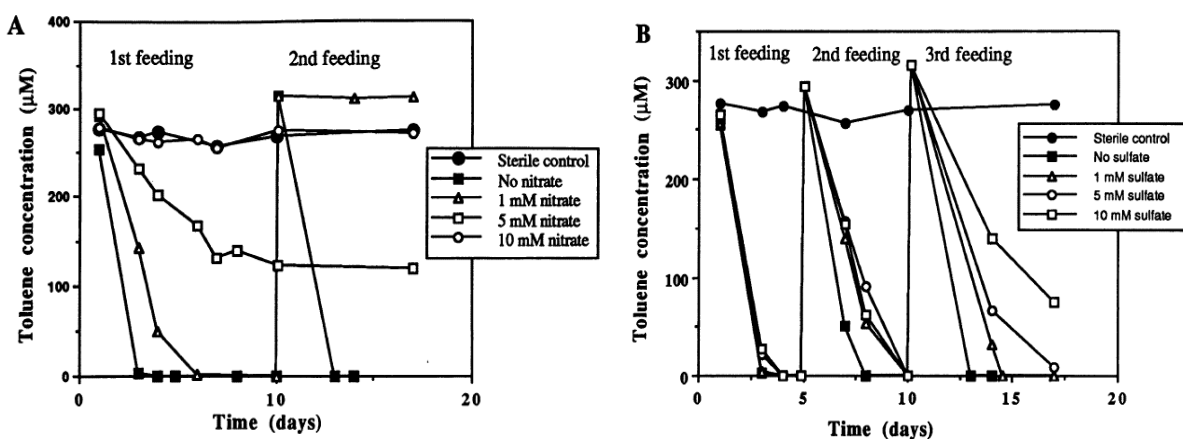
Reduction in toluene degradation rates, caused by excess sulfide concentrations, were mitigated in mixed cultures originating from fuel-contaminated soils in Maryland, by inclusion of iron (Beller and Reinhard, 1995). Providing FeSO<sub>4</sub> as a sulfate source, as opposed to MgSO<sub>4</sub>, not only maintained but enhanced toluene degradation rates, as sulfide produced by the cultures precipitated out as FeS, preventing accumulation of sulfide in the aqueous phase (Beller and Reinhard, 1995). The addition of the chelating agent, ethylenediaminetetraacetic acid (EDTA), provided supporting data consistent with the conclusion that iron amendments ease sulfide toxicity, not nutritional iron limitations; since the presence of EDTA with Fe<sup>2+</sup> had no enhancing effect on

toluene degradation, showing similar degradation results as cultures where iron was not provided and sulfide concentrations increased over time (Beller and Reinhard, 1995).

Studies at Stanford University utilized a mixed methanogenic inoculum from a stable consortium, originally enriched from sewage (Grbic-Galic and Vogel 1987). Noting that previous observations showed that toluene and benzene moderately inhibit methanogenesis, an initial lag phase was reported, in a pre-experimental period, of 4 and 16 days with 1.5 mM of both toluene and benzene, and of 15 mM toluene, respectively (Grbic-Galic and Vogel 1987). Further lag times were avoided by sufficiently acclimating culture to the substrates, over a 3-month period, so that transformation could start immediately (Grbic-Galic and Vogel 1987). The resulting data showed higher levels of labelled  $^{14}\text{CO}_2$  were produced from methyl labeled toluene than from ring-labeled toluene, suggesting that methyl carbon is likely converted to carbon dioxide and not methane, although  $\text{CH}_4$  was admittedly not measured (Grbic-Galic and Vogel, 1987). It was also noted that when added methanol was present in cultures, the transformation of toluene was slowed resulting in higher concentrations of toluene at the end of the 60-day incubation period (Grbic-Galic and Vogel 1987).

In 1994, further studies examined methanogenic cultures' ability to anaerobically degrade toluene. A culture was enriched by taking solids from a creosote-contaminated aquifer and refeeding active microcosms toluene and *o*-xylene (Edwards and Grbic-Galic 1994). The putative intermediates *p*-cresol and phenol were amended in some cases in an attempt to stimulate toluene degradation, however significantly long lag times were still observed prior to onset of toluene degradation; 100 days for toluene with *p*-cresol and 120 days for toluene without *p*-cresol (Edwards and Grbic-Galic 1994). For this methanogenic culture, the optimum pH for toluene degradation was determined to be near 6.0, while 75% of that degradation rate was achieved at pH

of 7.0 (Edwards and Grbic-Galic 1994). After two years of continuous enrichment, the culture's degradation rate had increased 10-fold primarily as a result of increased biomass concentration (Edwards and Grbic-Galic 1994). Alternate electron donors, nitrate and sulfate had an inhibitory effect (shown in Figure 2.4) (Edwards and Grbic-Galic 1994).



**Figure 2.4.** Inhibition of toluene degradation by alternative electron donors Nitrate (A) and Sulfate (B). [Figure reprinted from Edwards and Grbic-Galic (1994)]

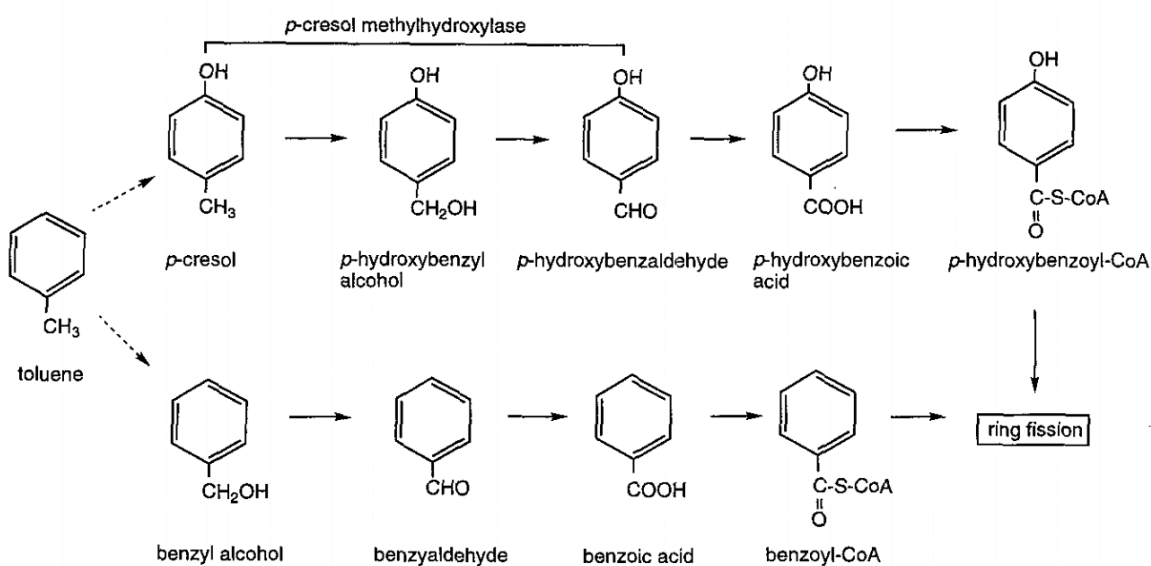
Several denitrifying, toluene degrading, bacteria have been identified, including but not limited to those within the genera of *Aromatoleum*, *Azoarcus*, *Dechloromaonas*, and *Thauera* (Altenschmidt and Fuchs, 1991; Hurek *et al.*, 1995; Rabus and Widdel 1995; Zhou *et al.*, 1995; Coates *et al.*, 2001; Shinoda *et al.*, 2004). There have also been a number of microorganisms reportedly linked to toluene degradation under sulfate-reducing conditions such as those in the genera *Desulfotomaculum* (Ficker *et al.*, 1999; Morasch *et al.*, 2004), *Desulfotignum* (Ommedal and Torsvik, 2007), and *Desulfosporosinus* (Liu, *et al.*, 2004; Sun and Cupples, 2012). Toluene degradation has also been noted under iron-reducing conditions by bacteria associated with the genera *Geobacter* (Kane *et al.*, 2002) and *Desulfuromonas* (Kim *et al.*, 2013) and by members of both *Methanosaeta* and *Methanospirillum* under methanogenic conditions (Ficker *et al.*, 1999).

In 1995, a review of toluene degradation under anaerobic conditions, utilizing information available at the time, put forth the proposed pathways shown in Figure 2.5 (Frazer *et al.*, 1995).



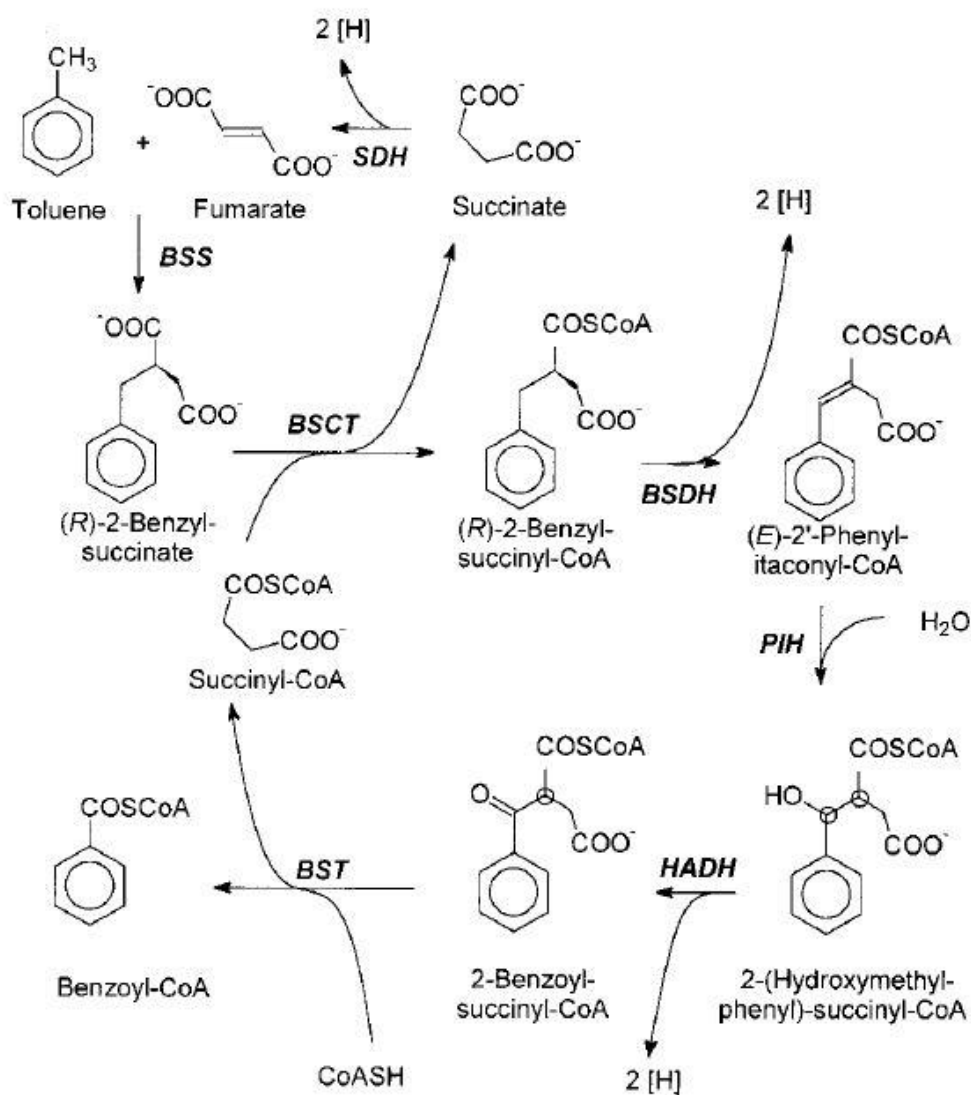
While it was noted that the pathways proposed were consistent with certain experimental observations, they acknowledged that definitive proof was not available for any of the routes summarized (Frazer *et al.*, 1995). Further investigations of anaerobic toluene catabolic pathways, in particular those involving the cell-free extracts of the denitrifying bacterial species *Thauera aromatica*, found that benzylsuccinate, of which fumarate was shown to be the cosubstrate during formation, was a true intermediate in the O<sub>2</sub>-independent oxidation of toluene to benzoyl-CoA (Biegert *et al.*, 1996). When (<sup>2</sup>H<sub>8</sub>)toluene was used as a substrate, analysis indicated that (<sup>2</sup>H<sub>7</sub>)benzylsuccinate was indeed formed from (<sup>2</sup>H<sub>8</sub>)toluene. In addition, experiments utilizing labeled benzylsuccinate found that a decrease in [<sup>14</sup>C]benzylsuccinate coincided with an increase in labeled benzoyl-CoA (Biegert *et al.*, 1996). In determining requirements for the formation of intermediates, results indicated that toluene was not oxidized to benzoyl-CoA and benzoate when either nitrate or coenzyme A were missing in the assay (Biegert *et al.*, 1996). These findings were further supported during biochemical and genetic characterization, which purified the enzyme responsible for catalyzing the reaction, benzylsuccinate synthase (Leutwein *et al.*, 1998). Furthermore, while cell extracts routinely catalyzed the fumarate-dependent formation of [<sup>14</sup>C]benzylsuccinate from [<sup>14</sup>C]toluene, the enzyme was rapidly inactivated by exposure to oxygen, and could not be reactivated upon transfer into anaerobic vials (Leutwein *et al.*, 1998). It was postulated that the four genes, designated bssDCAB, for benzylsuccinate synthase and the activating enzyme were organized as a single operon, for which transcription was induced by the presence of toluene; enzyme activity was only detected in the extracts of cells grown anaerobically on toluene but not in cells grown on other aromatic substrates (Leutwein *et al.*, 1998). Additional experimentation with cell extracts of *T. aromatica* indicate the pathway of benzylsuccinate oxidation required activation of the free acid to a CoA-thioester, catalyzed by succinyl-CoA-

dependent CoA-transferase, which would eventually lead to the proposed pathway depicted in Figure 2.6 (Leutwein and Heider, 1999).



**Figure 2.5.** Proposed pathways for toluene metabolism via methyl group hydroxylation of toluene (lower) or of *p*-cresol (upper) following the para-hydroxylation of the toluene ring. Enzyme activities in cell extracts have been demonstrated for all the transformations except those indicated with dashed line. [Reprinted from *Anaerobe*, 1 (6), Frazer, A.C., Coschigano, P.W., and Young, L.Y., Toluene Metabolism under Anaerobic Conditions, 293 – 303, Copyright 1995, with permission from Elsevier]

While the benzylsuccinate synthase, which catalyzes the addition of fumarate to the methyl carbon of toluene, has been previously reported in aerobic toluene-degrading, denitrifying, and sulfate-reducing bacteria, detection of high benzylsuccinate synthase was also reported in a highly enriched toluene-degrading, methanogenic culture (Beller and Edwards, 2000). The methanogenic culture utilized by Beller and Edwards (2000) was the same culture used in Edwards and Grbic-Galic (1994), which was originally obtained from a creosote-contaminated aquifer (Ficker *et al.*, 1999). The culture had been maintained in a batch mode and amended with approximately 1 mM toluene every 2 weeks and had consistently produced 85 to 100% of the theoretical methane yield (4.3 mol of methane/mol of toluene) for approximately 10 years with toluene as the only carbon source and electron donor (Beller and Edwards, 2000).



**Figure 2.6.** Proposed pathway of anaerobic toluene degradation. Enzymes: BSS, (R)-benzylsuccinate synthase; BSCT, succinyl-CoA:(R)-benzylsuccinate CoA-transferase; BSDH, (R)-benzylsuccinyl-CoA dehydrogenase; PIH, (E)-Phenylitaconyl-CoA hydratase; HADH, 3-hydroxyacyl-CoA dehydrogenase; BST, benzoylsuccinyl-CoA thiolase; SDH, succinate dehydrogenase. Chiral C atoms of as-yet-unknown configuration are indicated with circles [Figure and caption reprinted from Leutwein and Heider, 2001]

Previous genomic analysis of this culture indicated that it was dominated by two archaeal species presumed to play complimentary roles in the syntrophic culture (members of the genera *Methanosaeta* and *Methanospirillum*), and two eubacterial species, one belonging to the genus *Desulfotomaculum*, the other a eubacterial organism designated as OTU Eub-6 (Ficker *et al.*,

1999). It had been proposed that OTU Eub-6 represents the organism that initiated the degradation of toluene (Ficker *et al.*, 1999). Benzylsuccinate formation from toluene and fumarate accounted for >85% of toluene consumed in this methanogenic culture (Beller and Edwards, 2000). It was noted at the time “Benzylsuccinate, which is apparently the first metabolite of toluene mineralization in this culture is one of a number of candidates for interspecies metabolite transfer” (Beller and Edwards 2000).

In 2005, investigating possible *in situ* biotransformation of BTEX compounds at a gasoline spill, it was noted that while natural attenuation could be enhanced by the addition of sulfate and/or nitrate, due to the lack of groundwater flow at large spill sites, the supply of electron acceptors can be constrained in a manner such that methanogenesis may be the only possible mechanism of removal (Reinhard *et al.*, 2005). In the study, BTEX and other organics as well as existing electron acceptors (nitrate and sulfate) and dissolved oxygen and residual BTEX was removed with a helium-purged gas-stripping tower (Reinhard *et al.*, 2005). After returning the waters ionic strength to levels approximately equal to that of the original groundwater, the experimental volume was augmented with benzene, toluene, ethylbenzene, and *o*-xylene (Reinhard *et al.*, 2005).

All experiments showed that toluene and *o*- and *m*-xylenes, when present, were rapidly transformed within the first 25 days and bromide tracer concentrations indicated no dilution from groundwater crossflow (Reinhard *et al.*, 2005). While toluene degradation rates were initially slower, there was no observed lag phase (Reinhard *et al.*, 2005). It was noted that while the comparison of transformation rates calculated for all the BTEX compounds fell in the lower ranges to those values previously reported in the literature under methanogenic conditions, it was expected since the reported values came from laboratory enrichment cultures and would not necessarily represent values for *in situ* transformation (Reinhard *et al.*, 2005).

Enriched anaerobic cultures taken from petroleum contaminated sediment in an Amsterdam harbor indicated the ability to biologically oxidize toluene to CO<sub>2</sub> when coupled with humus respiration (Cervantes *et al.*, 2001). In the study, highly purified soil humic acids (HPSHA) and quinone moiety of hummus, anthraquinone-2,6-disulfonate (AQDS) were utilized as terminal electron acceptors allowing for the recovery of 50 and 85% of labeled <sup>13</sup>C toluene as <sup>13</sup>CO<sub>2</sub> (Cervantes *et al.*, 2001). In the absence of humic acids, the culture supported toluene degradation when utilizing nitrate and Mn(IV) as electron acceptors, however showed no degradation of toluene under sulfate-reducing, methanogenic, or iron-reducing conditions after 4-month incubations (Cervantes *et al.*, 2001). However, stimulation of toluene degradation occurred when sediment mixtures were amended with goethite (FeOOH, 50 mM) with a sub-stoichiometric amount of humic acids (2 g/L) (Cervantes *et al.*, 2001). While the amount of humic acids could only account for biodegradation of 1.7% of toluene, when low levels of humic acid was added, more than 65% of toluene had been depleted after 11 weeks; negligible conversion of toluene was reported in goethite supplemented cultures when humic acids were omitted (Cervantes *et al.*, 2001). Furthermore, availability of humic acids also reduced the lag time of cultures utilizing Mn (IV) as an electron acceptor (Cervantes *et al.*, 2001). While it was noted that Goethite was not utilized directly as an electron acceptor, conversion of toluene was made possible only by supplementing goethite-containing cultures with sub-stoichiometric levels of humic acid, leading to the postulation that stimulation can only be accounted for by a chelating effect of humic acids with Fe(III) or a redox-mediating effect (Cervantes *et al.*, 2001). A list of various reactions involved in the degradation of toluene and the respective Gibbs free energy calculated by Cervantes *et al.* (2001) is presented in Table 2.4.

**Table 2.4.** Thermodynamic comparisons of biodegradation of toluene with alternative electron acceptors. (Table reprinted from Cervantes *et al.*, 2001, whom utilized additional data collected from Langenhoff, 1997; Sober, 1970; and Thauer *et al.*, 1977)

Reaction	$\Delta G^{\circ}$ (kJ/mol)
$C_7H_8 + 36 Fe^{3+} + 21 H_2O \longrightarrow 36 Fe^{2+} + 43 H^+ + 7 HCO_3^-$	-3,629.6
$C_7H_8 + 7.2 NO_3^- + 0.2 H^+ \longrightarrow 3.6 N_2 + 0.6 H_2O + 7 HCO_3^-$	-3,554.8
$C_7H_8 + 18 MnO_2 + 18 H_2CO_3 \longrightarrow 7 CO_2 + MnCO_3 + 22 H_2O$	-3,358.8
$C_7H_8 + 36 FeO(OH) + 36 H^+ \longrightarrow 7 CO_2 + 36 Fe(OH)^+ + 22 H_2O$	-1,443.6
$C_7H_8 + 18 AQDS + 21 H_2O \longrightarrow 18 AH_2QDS + 7 H^+ + 7 HCO_3^-$	-319.7
$C_7H_8 + 4.5 SO_4^{2-} + 3 H_2O \longrightarrow 4.5 HS^- + 2.4 H^+ + 7 HCO_3^-$	-205.2
$C_7H_8 + 7.5 H_2O \longrightarrow 4.5 CH_4 + 2.5 H^+ + 2.5 HCO_3^-$	-130.7

While multiple anaerobic toluene biotransformation pathways have been postulated (see Figure 2.5 and 2.6), more recent reports have indicated that abiotic formation of cresols and phenol are also possible. While studying a methanogenic toluene-degrading mixed culture originating from a gas condensate-contaminated aquifer, it was noted that formation of cresols and phenol, among other hydroxylated compounds, had formed in abiotic controls; particularly when those controls were exposed to oxygen prior to extraction (Fowler *et al.*, 2012). Furthermore, while studying anaerobic degradation of benzene in an iron-reducing enrichment culture, a similar phenomenon was observed (Kunapuli *et al.*, 2008). Here it was reported that phenol was formed abiotically by autoxidation of benzene during the sampling, leading to the hypothesis that ferrous iron reacting with oxygen formed hydroxyl radicals, which in turn formed abiotically-produced phenol when these radicals reacted with benzene (Kunapuli *et al.*, 2008). This prompted Fowler *et al.* (2012) to note that care should be taken when extracting highly reduced anaerobic cultures, as “...exposure to oxygen must be minimized, and even then, the origin of compounds observed in metabolite analysis should be confirmed using isotope labelling...” (Fowler *et al.*, 2012). However, they were able to confirm benzylsuccinate as a veritable metabolite as it was observed to be  $^{13}C$  labelled in incubations with  $^{13}C_7$  toluene (Fowler *et al.*, 2012). They also noted that a

BssA gene fragment was amplified from this enrichment culture using PCR, supporting fumarate addition as a key mechanism for toluene degradation under methanogenic conditions (Fowler *et al.*, 2012).

### 3 STUDIES AIMED AT UNDERSTANDING THE POTENTIAL FOR BIOLOGICALLY-MEDIATED TOLUENE FORMATION

This chapter contains a description of experiments conducted to evaluate the potential for toluene biogenesis by microbial populations in the distal plume area of a south Louisiana Superfund site. The chapter also presents data from the field sites from which samples were collected.

#### 3.1 Site Location and Background

For the purposes of this thesis, the “distal plume area” refers to an area of an aquifer approximately two miles downgradient from where petrochemicals, including a variety of free-phase chlorinated solvents, were disposed of by direct discharge to unlined earthen lagoons between 1961 and 1980. In an effort to halt further downgradient migration of aqueous-phase pollutants, a system of wells was installed in late 2011 and early 2012 to allow subsurface injection of electron donors to facilitate *in situ* reductive dechlorination. Locations of selected wells sampled during the course of experiments conducted in the course of research described in this thesis are shown in Table 3.1.

**Table 3.1.** Locations of groundwater wells sampled during the course of this research

Class-V Injection DTZ Well No.	SP013	SP024
Well serial number	974196	974206
Date installed	1-18-12	1-13-12
Latitude (NAD 83)	30° 35' 41.0796"	30° 35' 43.1102"
Longitude (NAD 83)	91° 14' 43.4671"	91° 14' 43.3337"
Depth (feet bgs <sup>a</sup> )	93 feet	113 feet
Screened interval (feet bgs <sup>a</sup> )	51-91 feet	55-119 feet
Top of casing elevation (NAVD 88)	75.61 feet	76.94 feet

<sup>a</sup> bgs = below ground surface

The subsurface injection of molasses via injection well extraction and recirculation in the distal plume treatment zone commenced in June 2013, and again in August 2015. Dates are displayed in Table 3.2.



**Table 3.2.** Molasses injection dates for Well SP024.

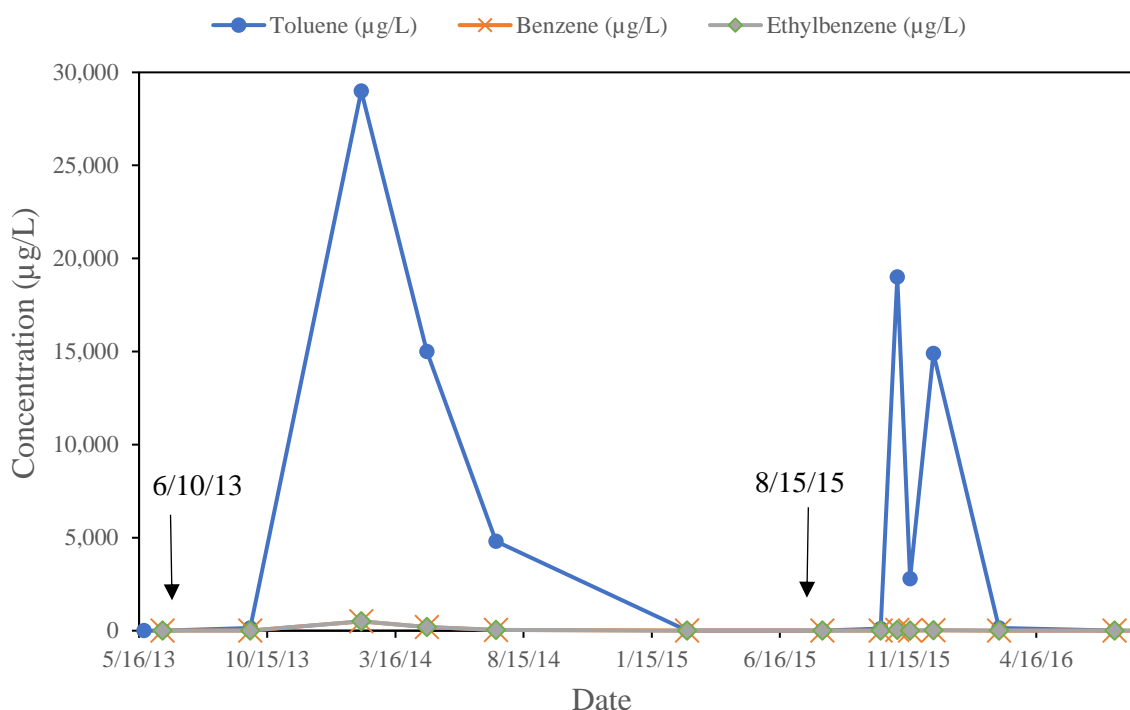
Well SP024
6/10/13 – 6/13/13
8/15/15** (approx.)

### 3.2 Site Monitoring Data

Groundwater samples collected from various injection wells and monitoring wells at multiple times were analyzed for VOCs and geochemical parameters. Analyses were conducted at a certified analytical laboratory (Gulf Coast Analytical, Baton Rouge, LA). As with groundwater monitoring data previously reported for the same site by Bowman *et al.* (2006); concentrations of volatile organic compounds were measured using US EPA method 624 (Bowman *et al.*, 2006). Dissolved ethene, ethane, and methane were measured using method RSK 175 (Bowman *et al.*, 2006). Nitrate and nitrite were measured using US EPA method 353.2 (Bowman *et al.*, 2006). Chloride was measured using US EPA method 252.2 (Bowman *et al.*, 2006). Sulfate was measured by ion chromatography using US EPA method 300.0 (Bowman *et al.*, 2006). Sulfide was measured using US EPA method 376.2 (Bowman *et al.*, 2006). Ferrous iron was measured using US EPA method 3500-Fe D (Bowman *et al.*, 2006). Total organic carbon was measured using US EPA method 5310B (Bowman *et al.*, 2006). Detailed descriptions of the US EPA analytical methods referenced above are available elsewhere (National Environmental Index, <http://www.nemi.gov/>). Tabulated data are shown in Appendix B.

Well SP024 received an injection of molasses on June 10, 2013. Analysis on June 10, 2013 had toluene levels less than 0.001 mg/L. Over three months later, on September 25, toluene levels were still relatively low at 0.139 mg/L. However, the following analysis on February 4, 2014 indicated toluene had increased dramatically to 29.0 mg/L (Figure 3.1). While toluene concentrations receded in the months following, concentrations as high at 15.0 mg/L were still recorded 10 months after the molasses injection.

Analysis of SP024 groundwater two months after the second injection of molasses in August 2015 again indicated relatively low levels of toluene (0.103 mg/L). Then on November 3, 2015, nearly three months after the injection, toluene levels again rose dramatically to 19.0 mg/L remaining as high as 14.9 mg/L on December 16, four months after initial injection. By March 3, 2016, 6 months after injection, toluene levels had receded once again, to 0.146 mg/L.



**Figure 3.1.** BTE (Benzene, Toluene, Ethylbenzene) concentration over time in groundwater sampled from well SP024. Arrows denote molasses injection dates. For compounds not detected (benzene, ethylbenzene), concentration of their respective detection limit is plotted.

### 3.3 Experimental Methods

The groundwater was collected in sterile 1-L nominal capacity glass media bottles filled leaving little or no gas headspace. Immediately after collection of samples for microcosms and enrichment cultures, additional samples were collected by NPC Services personnel for analysis of VOCs and geochemical parameters (analyses performed at Gulf Coast Analytical – see Appendix B1 for results).

All enrichment cultures used media and/or groundwater aseptically dispensed under anaerobic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) supplied with an anaerobic gas mix comprised of 80% N<sub>2</sub>, 10% H<sub>2</sub>, and 10% CO<sub>2</sub>. Glass serum bottles (Wheaton) were autoclaved (121°C, 15 psi, 20 min) prior to use and were sealed with sterile butyl rubber stoppers and aluminum crimp caps prior to removal from the anaerobic chamber. Unless otherwise noted, the gas headspace of bottles dispensed and sealed in the anaerobic chamber were purged for a minimum for 25 seconds with a filter-sterilized gas mixture comprised of 5% CO<sub>2</sub> balance N<sub>2</sub> under 10 psi pressure with 20-gauge hypodermic needles. An effort was made to maintain a similar ratio of liquid to gas head space in each experiment, regardless of bottle size. Experiments utilizing 25 mL serum bottles had 15 mL liquid and 10 mL headspace. Those using 162 mL bottles, had 100 mL liquid and 62 mL headspace.

### 3.3.1 Analytical Techniques

Toluene concentrations were measured using an Agilent Technologies Gas Chromatograph (GC) Model 7820A with a flame ionization detector (FID) and an Agilent (123-1364) DB-624 capillary column (60 m × 0.32 mm × 1.80 µm). The GC was programmed starting with a five-minute hold at 40° C (minutes 0-5), a 20° C/minute temperature ramp for 11 minutes (minutes 5-16), and a 3-minute hold at 260° C (minutes 16-19).

Gas headspace samples were introduced to the GC via splitless injection using a Pressure-Lok® 100-µl gas-tight syringe from VICI Precision Sampling Inc. Aqueous-phase samples were introduced to the GC utilizing a Teledyne Tekmar AQUAtek 100 auto sampler in conjunction with a Teledyne Tekmar Purge and Trap (Model #: 14-9800-100).

### 3.3.2 Media Preparation

Initial experiments utilized anaerobic TP medium (Fischer-Romero *et al.*, 1996) as previously described for use in the isolation and cultivation of *Tolumonas auensis* TA4<sup>T</sup> but with the modification that resazurin was added to a final concentration of 1 mg/L to serve as a redox indicator, zinc chloride in the trace element solution was replaced with an equal molar concentration of zinc acetate, and the glucose concentration was increased to 2 g/L (from 1 g/L in the medium described by Fischer-Romero *et al.* (1996).

After preliminary experiments utilizing TP medium, as described by Fischer-Romero *et al.* (1996), failed to accumulate toluene, the medium was further modified by removal of Na<sub>2</sub>S·9H<sub>2</sub>O. This formulation, hereafter referred to as Modified TP medium, contained the following constituents (per liter): KH<sub>2</sub>PO<sub>4</sub>, 0.25 g; NH<sub>4</sub>Cl, 0.34 g; KCl, 0.34 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.25 g; glucose, 2 g; NaHCO<sub>3</sub>, 1.5 g; phenylalanine, 132 mg; resazurin, 1 mg; yeast extract, 10 mg; Vitamin B<sub>12</sub>, 0.02mg; FeCl<sub>2</sub>·4H<sub>2</sub>O, 1.5 mg; H<sub>3</sub>BO<sub>3</sub>, 0.006 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.1 mg; CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.19 mg; Zinc Acetate ·2H<sub>2</sub>O, 0.144 mg; NiCl<sub>2</sub> ·6H<sub>2</sub>O, 0.024 mg; CuCl<sub>2</sub> ·2H<sub>2</sub>O, 0.002 mg; Na<sub>2</sub>MoO<sub>4</sub> ·2H<sub>2</sub>O, 0.036 mg. A detailed protocol for how this anaerobic medium was made is provided in Appendix A1.

In an attempt to establish a growth medium to enable propagation of a toluene-producing culture, the Modified TP medium was, in some cases, further altered by substituting phenylalanine for phenylacetic acid, as well as the addition of, and substituting for glucose, of various alternative carbon sources as further described in Section 3.3.4.2.

Later in the research process, the propagation of a toluene-producing culture employed a growth medium as described by Zargar *et al.* (2016). The medium as described by Zargar *et al.* (2016) (full protocol outlined in Appendix A2) contained the following constituents (per liter):

KH<sub>2</sub>PO<sub>4</sub>, 0.25 g; NH<sub>4</sub>Cl 0.34 g; KCl 0.34 g; MgCl<sub>2</sub>·6H<sub>2</sub>O 1 g; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g; CaCl<sub>2</sub>·2H<sub>2</sub>O 0.125 g; Sodium HEPES 4.685 g; glucose 1.0 g; yeast extract, 10 mg; phenylacetic acid, 27.2 mg; vitamin B<sub>12</sub>, .029 mg; FeSO<sub>4</sub>·7H<sub>2</sub>O, 1.05 mg; MnCl<sub>2</sub>·4H<sub>2</sub>O 0.05mg; CoCl<sub>2</sub>·6H<sub>2</sub>O 0.095mg; ZnCl<sub>2</sub>, 0.035mg; NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.012, CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.001 mg; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.018 mg; H<sub>3</sub>BO<sub>3</sub>, 0.003 mg. After medium was completed, 7.1 mL (1 M HCl) was added to achieve a pH of 7.1-7.2, approximately.

### 3.3.3 Potential for Toluene Production

To investigate the potential for microbial communities to biologically toluene production in the groundwater rather than a defined growth medium, groundwater collected from Well SP024 on November 18, 2015 was amended with various combinations of glucose and/or phenylalanine. Prior to the addition of amendments, the groundwater samples were purged with a filter-sterilized gas mixture comprised of 5% CO<sub>2</sub> and 95% N<sub>2</sub> for 10 minutes in an effort to remove toluene present in the aqueous phase. After purging, each bottle received one of five treatments. The first treatment received 11 mL of glucose solution (100 g/L, see Appendix A1 for preparation protocol) to 550 mL of groundwater, resulting in a final glucose concentration of 2000 mg/L). A second treatment received 11 mL of phenylalanine solution (6.6 g/L, see Appendix A1 for preparation protocol) to 550 mL of groundwater, resulting in a final amended phenylalanine concentration of 132 mg/L). A third treatment received 11 mL of glucose solution and 11 mL of phenylalanine solution to 550 mL of groundwater, resulting in final amended concentrations of 2000 mg/L glucose and 132 mg/L phenylalanine. The fourth treatment, a control, received no additional amendments. The final treatment, received no additional amendments and was then autoclaved to serve as an abiotic negative control. Each treatment was inverted several times to provide mixing. For each treatment, 15 mL aliquots were aseptically dispensed into 34 separate, sterile, glass 25-

mL serum bottles under anaerobic conditions. Headspace of each bottle was then purged for 30 seconds at 10 psi with a filter-sterilized gas mixture comprised of 5% CO<sub>2</sub> balance N<sub>2</sub>. Bottles were incubated in the dark without mixing at ambient laboratory temperature (approximately 22°C). At regular intervals, triplicate bottles were sacrificed for analysis of aqueous-phase toluene concentrations via purge-and-trap gas chromatography as described in Section 3.3.1.

### 3.3.4 Enrichment, Propagation, and Testing of Various Carbon Sources and Phenyl Containing Precursor concentrations

#### 3.3.4.1 Enrichment Cultures

To establish enrichment cultures, groundwater was collected from various sample wells at over an 18-month time span. Modified TP medium, utilizing various carbon sources and either phenylalanine or phenylacetic acid, was inoculated with groundwater at 5% (volume/volume) in 25-mL serum bottles with a total of 15 mL medium/inoculum and 10 mL gas headspace. Bottles were incubated in the dark without mixing at ambient laboratory temperature (approximately 22°C). At regular time intervals, replicate bottles were sacrificed for analysis of aqueous-phase toluene concentrations via purge-and-trap gas chromatography as described in Section 3.3.1.

#### 3.3.4.2 Testing of Various Carbon Sources

Effects of various carbon sources on toluene production were determined by utilizing as an inoculum what would later be referred to as “October 2015 microcosm”, which was previously shown to produce toluene in Modified TP medium. The culture to be used for inoculation of experiments testing various carbon sources, had been stored at 4°C for approximately 9 months, since it was initially collected from Well SP024 on 10/14/15. The Modified TP medium formulation was used as a basis, but with varying carbon sources, and in one series altering the precursor. That series replaced phenylalanine with phenylacetic acid (82 mg/L) while all remaining series contained phenylalanine (132 mg/L) as a precursor. Carbon sources used

included: Lignin (600 mg/L) both with and without glucose (2,000 mg/L), ‘Avicel’ (cellulose) (500 mg/L) both with and without glucose (2,000 mg/L), and agricultural-feed grade molasses (Westway) both with and without Iron (III) Nitritotriacetate (0.7 mM).

#### 3.3.4.3 Propagation Experiments

Replicates from experiments that accumulated an appreciable concentration of toluene ( $\geq 20$  mg/L over initial inoculated concentration) were used to inoculate either Modified TP medium at varying glucose concentrations (0, 0.3, and 2 g/L) or, in later experiments, medium as described by Zargar *et al.* (2016) (1 g/L) in attempts to propagate an enrichment culture. Modified TP medium utilized phenylalanine and medium as described by Zargar *et al.* (2016) utilized phenylacetic acid as precursors. Bottles of varying volumes were incubated in the dark without mixing at ambient laboratory temperature (approximately 22°C). At regular intervals, replicate bottles were sacrificed for analysis of aqueous-phase toluene concentrations via purge-and-trap gas chromatography as described in Section 3.3.1.

#### 3.3.4.4 Varying Precursor Concentration Experiments

Using an enrichment culture observed to consistently accumulate toluene, and propagated through 3 generations in growth medium as described by Zargar *et al.* (2016), an experiment varying precursor concentration with 5% inoculum (vol./vol.) was established based on the medium as described by Zargar *et al.* (2016). One series was set up with varying concentration of phenylalanine, while another was set up with varying concentrations of phenylacetic acid. Precursor (i.e., phenylacetic acid or phenylalanine) stock solutions were established at 3000, 2250, 1500, 1125, 750, 600, 450, 300, 150 and 75 mg/L. Final concentrations of each precursor following addition to the completed medium were 5, 10, 20, 30, 40, 50, 75, 100, 150, 200, 300, and 400 mg/L; being reached by injecting 1 mL of stock solution into each bottle, with the

exception of those with precursor concentrations of 300 and 400 mg/L. Due to solubility constraints, final concentrations for 300 and 400 mg/L were achieved by injecting 1.5 mL of the highest concentration (3000 mg/L) to achieve 300 mg/L and 2 mL to achieve 400 mg/L. It should be noted that while adding precursors from stock solutions were taken into consideration when preparing the medium, it was not accounted for the additional volume for two highest concentrations. As a result, the remaining constituents' concentrations would have been reduced by ~6% for the 400 mg/L series, and ~3% for the 300 mg/L series. Four replicates were established at each concentration level. Bottles were then incubated in the dark without mixing at ambient laboratory temperature (approximately 22°C). Replicates from each series were analyzed for aqueous-phase toluene concentrations after 28 days incubation.

To facilitate mass balance calculations, the amount of toluene per bottle was calculated using an assumed dimensionless Henry's Law Constant of 0.209 [=0.00506 atm·m<sup>3</sup>/mol] (Peng and Wan, 1997). [<https://www3.epa.gov/ceampubl/learn2model/part-two/onsite/esthenry.html>]

### **3.4 Results and Discussion**

#### **3.4.1 Microcosm Experiments and Enrichment Cultures Using Groundwater from Well SP024 Sampled October 14, 2015**

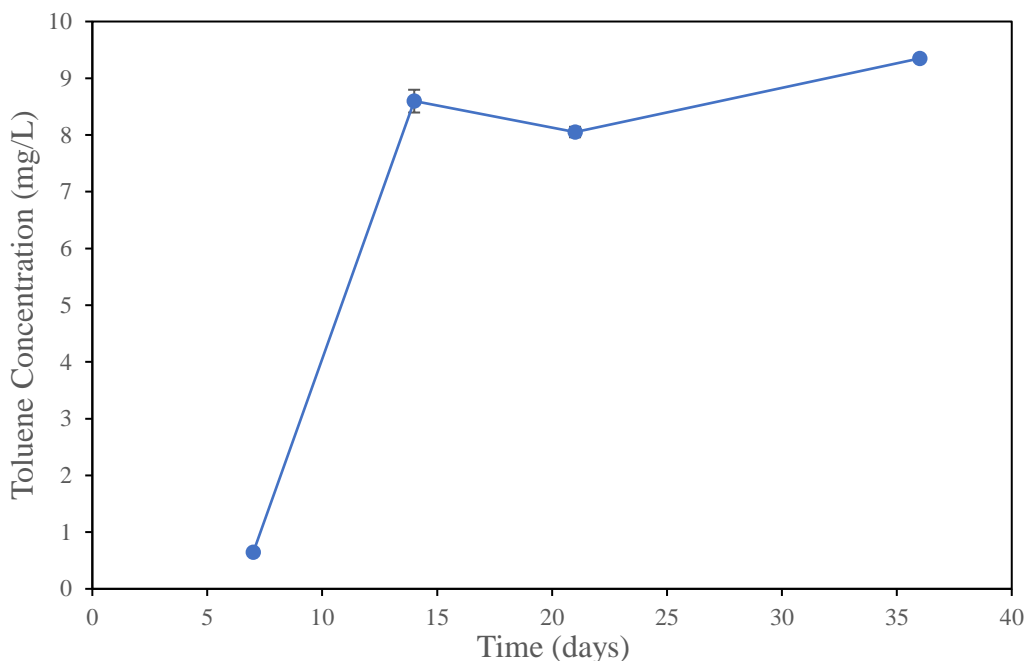
Groundwater collected from well SP024 on October 14, 2015 was used to establish microcosm experiments as well as inoculate enrichment cultures. After transport to the LSU laboratory, the groundwater sample bottles were transferred into an anaerobic chamber. For the microcosm experiment, 100 mL aliquots of groundwater were dispensed into each of eight sterile, 160 mL glass serum bottles that were capped with butyl rubber stoppers and aluminum crimp caps with no additional media supplements. For enrichment culture experiments, 400 mL aliquots of TP medium, with the formulation of Fischer-Romero *et al.* (1996) with exceptions as previously noted in Section 3.3.2, prepared at double-strength concentration was mixed with an equal volume



of groundwater. This resulted in final medium concentrations as described by Fischer-Romero *et al.* (1996) [aside from minor exceptions as noted in Section 3.3.2]. 100 mL aliquots of the growth medium/groundwater mixtures were dispensed into sterile 160 mL serum bottles that were capped with butyl rubber stoppers and aluminum crimp caps. All bottles were incubated at ambient laboratory temperature (approximately 22°C).

At 7-day intervals, headspace gas samples from two microcosm replicates and two enrichment culture replicates were analyzed by gas-phase chromatography to quantify toluene. For the groundwater-only microcosms, the gas-phase toluene headspace concentration increased from  $0.6 \pm 0.1$  mg/L on Day 7 to  $8.6 \pm 0.4$  mg/L on Day 14 (Figure 3.2). Using Henry's Law to estimate the aqueous concentration gave calculated aqueous-phase concentrations of  $2.9 \pm 0.5$  mg/L on Day 7 increasing to  $41.1 \pm 2.0$  mg/L on Day 14. Subsequent replicates analyzed on Day 21 showed an average gas-phase concentration of  $8.1 \pm 0.2$  mg/L. This corresponds to an aqueous-phase toluene concentration calculated using Henry's Law to be  $38.7 \pm 1.0$  mg/L. Following analysis, microcosm replicates were stored at 4°C.

To confirm the magnitude of toluene production and directly measure the aqueous-phase toluene concentrations in microcosms, on Day 21, liquid aliquots from two microcosm bottles were transferred to 40 mL VOA vials, sealed with Teflon-lined septa, and then were analyzed by EPA Method 8260B at a certified analytical lab (Gulf Coast Analytical Laboratories, Baton Rouge, LA). Results of the two replicates showed aqueous-phase toluene from Day 21 were 37.2 and 39.7 mg/L, consistent with the values calculated based on measured gas-phase headspace concentrations and dimensionless Henry's Law Constant of 0.209. Benzene, ethylbenzene, and xylenes were below detection in the aqueous-phase samples analyzed on Day 21 and were not detected in any of the gas-phase analyses.



**Figure 3.2.** Gas-phase concentration of toluene over time in microcosms established using groundwater from Well SP024 collected on 10/14/15.

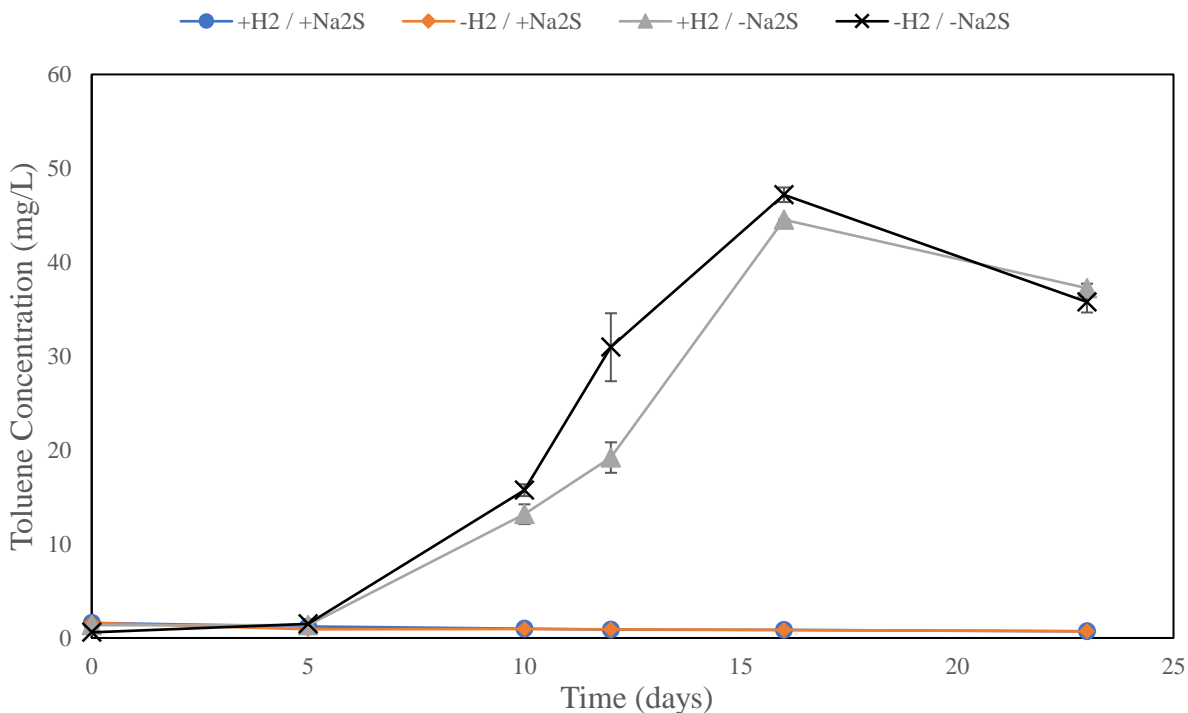
To assess whether the observed toluene accumulation in the laboratory microcosms (Figure 3.2) correlated with toluene accumulation in the site groundwater, groundwater from well SP024 was collected by NPC Services personnel on November 3, 2015 (which was Day 19 of the laboratory microcosm experiment) for analysis using EPA method 8260B (analyzed by GCAL). Analysis indicated that corresponding *in situ* aqueous-phase toluene concentrations had increased from 0.103 mg/L on October 14, 2015 (Day 0 of the laboratory incubation) to 19.0 mg/L on November 3, 2015 (Day 19 of the laboratory incubation) [tabulated data and other geochemical parameters are shown in Table B7 of Appendix B]. As shown previously in Figure 3.1, the elevated toluene concentration in the SP024 well persisted over time before decreasing, with 14.9 mg/L toluene present in samples collected on December 16, 2015 before decreasing to 0.146 mg/L on March 3, 2016 and 0.003 mg/L on July 18, 2016 (tabulated data in Table B7). During this time interval, benzene and ethylbenzene were below detection in the SP024 groundwater (with detection limits ranging from 0.2 to 40 µg/L as noted in Table B7).

The rapid increase in toluene concentration measured in the SP024 groundwater (field measurements) and microcosms (laboratory experiments) occurred in the timeframe of two to three months after the most recent subsurface injection of molasses in well SP024. The toluene concentration increase observed in laboratory microcosms can exclude the possibility that the toluene concentration increase observed in the field data was due to advection or other external introduction during this time interval. Additionally, toluene was not detected in the upgradient well SBP-089 sampled on October 14, 2015 ( $<1\ \mu\text{g/L}$ ).

While toluene accumulated to high levels in the groundwater microcosm serum bottles (groundwater only), no toluene was detected in the gas headspace analysis for enrichment cultures prepared using the double-strength TP medium in any of the replicates analyzed over the 28-day incubation time following inoculation with the same groundwater.

In an effort to determine if the presence of sulfide as a reducing agent in the TP medium or the presence of  $\text{H}_2$  in the gas headspace of enrichment culture serum bottles were potential reasons why toluene was not produced in the enrichment cultures but was produced in the groundwater-only microcosms, groundwater microcosms analyzed on Day 21 were used to inoculate four different treatments. This experiment utilized a series containing TP medium, as described by Fischer-Romero *et al.* (1996) with  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  at a final concentration of 5 mM, and a Modified TP medium (without  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ ); both series supplied with phenylalanine and glucose at 132 and 2,000 mg/L, respectively. Both series utilized 25-mL serum bottles with 10 mL of gas headspace and were inoculated with 5% (vol./vol.) with groundwater microcosms analyzed on Day 21. A portion of the replicate serum bottles with and without sulfide as a reducing agent were purged with a gas mixture containing 5%  $\text{CO}_2$  balance  $\text{N}_2$ , while the remaining bottles from each treatment were purged with a gas mixture consisting of 10%  $\text{CO}_2$ , 10%  $\text{H}_2$ , balance  $\text{N}_2$ .

After an initial lag phase, there was a marked increase in toluene over time in bottles with both gas headspace compositions for media prepared without Na<sub>2</sub>S. Results presented in Figure 3.3 display an average of toluene concentration of replicates analyzed over time. Toluene concentrations of  $15.7 \pm 0.6$  mg/L were observed in replicates of the series without Na<sub>2</sub>S/without H<sub>2</sub>, 10 days after inoculation. The highest toluene concentrations observed were recorded on Day 16 of  $47.2 \pm 0.8$  mg/L. In series without Na<sub>2</sub>S/with H<sub>2</sub>, toluene concentrations reached  $13.7 \pm 0.01$  mg/L, 10 days after inoculation, with the highest concentration on Day 16 of  $44.5 \pm 0.01$  mg/L. The presence of H<sub>2</sub> gas in the headspace did not appear to have a dramatic impact on accumulation of toluene. Toluene levels in either series containing Na<sub>2</sub>S did not show an increase in toluene concentration over time, and in some cases slightly decreased. Analysis of negative controls, which consisted of un-inoculated growth medium, showed no measurable concentration of toluene ( $<0.1$  mg/L).



**Figure 3.3.** Toluene concentration as a function of time in time-series experiment utilizing Modified TP medium with and without Na<sub>2</sub>S, with and without H<sub>2</sub> in the gas headspace.

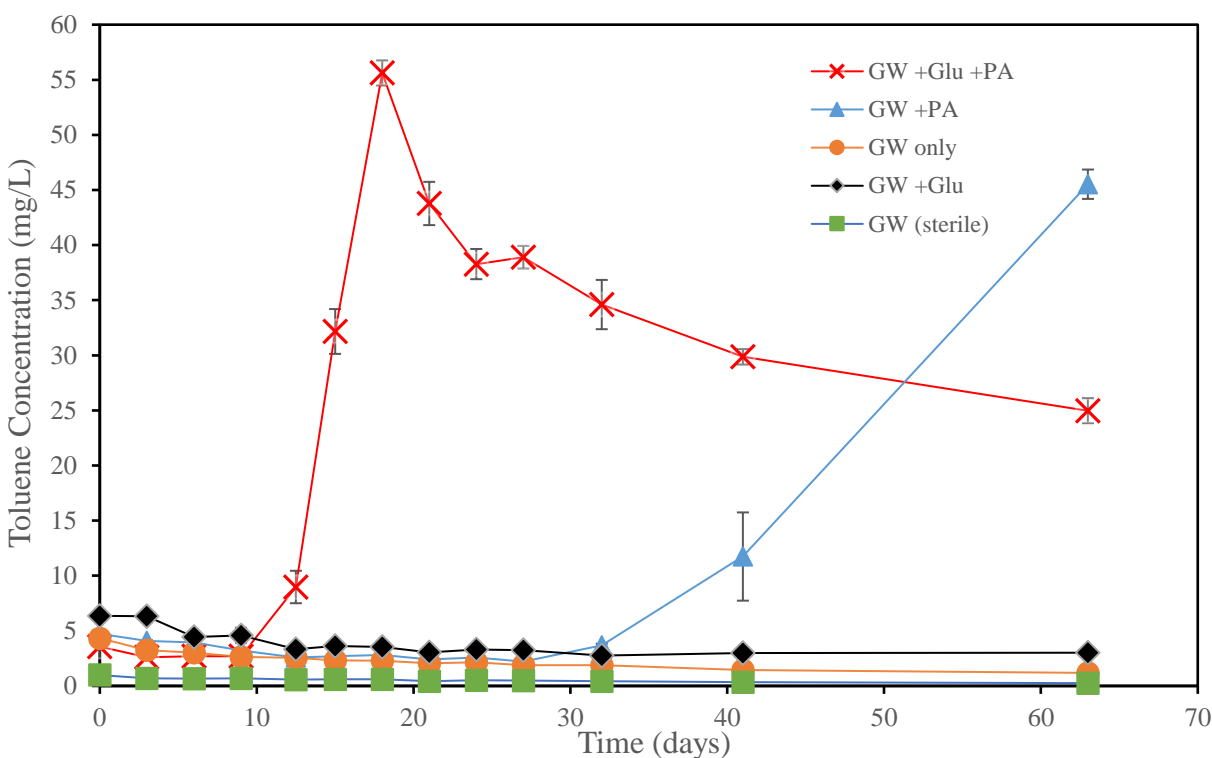
Experiments to further propagate the toluene-producing culture, following the incubation period depicted in Figure 3.3, was attempted in Modified TP medium (without sulfide) in two consecutive experiments; however, toluene did not accumulate to an appreciable level in any replicates analyzed (data not shown). An additional attempt to propagate the toluene-producing culture utilizing Modified TP medium amended with methionine to a final concentration of 200 mg/L also did not accumulate toluene (data not shown).

#### 3.4.2 Microcosms and Enrichment Cultures Established Using Groundwater from Well SP024 Sampled November 18, 2015

Groundwater collected from Well SP024 on November 18, 2015 was aseptically transferred to glass serum bottles (160 mL) which were sealed with butyl rubber stoppers and aluminum crimp seals. One series of replicate bottles contained groundwater amended with both glucose and phenylalanine from concentrated stock solutions. Other series of replicate bottles contained groundwater amended with only glucose or only phenylalanine. Still other replicate bottles contained groundwater that were not amended with any supplements. A portion of these replicates not receiving any amendments (i.e., groundwater only) were autoclaved to serve as abiotic controls. For groundwater that was amended with both phenylalanine and glucose, a distinct increase in toluene concentration was observed by Day 12 (see Figure 3.4), when the toluene concentration reached  $9.0 \pm 1.5$  mg/L (mean  $\pm$  standard deviation). The highest toluene concentrations,  $55.6 \pm 1.1$  mg/L, were recorded on Day 18 after which time toluene concentrations slowly declined.

The series containing phenylalanine without addition of glucose also exhibited considerable toluene accumulation but with a much longer lag period and slower rate of accumulation. A noticeable increase in toluene concentration was not observed until replicates sacrificed 32 days after inoculation (Figure 3.4). This was further confirmed after subsequent

analysis showed  $13.0 \pm 4.4$  mg/L and  $50.5 \pm 1.5$  mg/L on Days 41 and 63, respectively. All series without added phenylalanine showed no appreciable accumulation in toluene concentration, and in most cases observed concentrations of toluene decreased, albeit very slightly, over time (Figure 3.4).



**Figure 3.4.** Toluene concentration as a function of time in time-series experiment utilizing amended groundwater collected from SP024 on November 18, 2015.

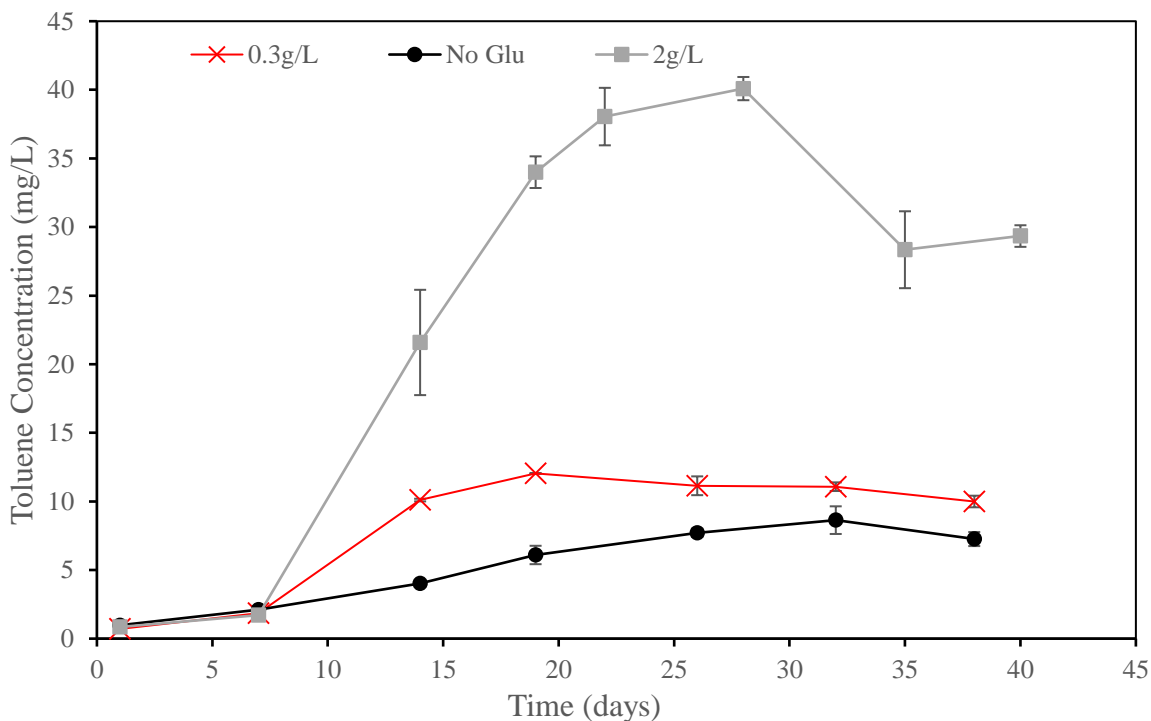
The fact that both series of enrichments amended with phenylalanine (with and without glucose) eventually produced toluene at relatively high and similar concentrations, while enrichments without phenylalanine did not accumulate toluene, suggests that access to a sufficient precursor could be a limiting factor. That toluene accumulation occurred with a shorter lag period and higher rate in phenylalanine-supplemented bottles with glucose than without, clearly demonstrates that glucose impacted the process. The fact that groundwater-only microcosms established from groundwater collected on November 18, 2015 did not accumulate toluene, in sharp contrast to those established using groundwater collected on October 15, 2015, may reflect

that the precursor(s) in the groundwater were depleted *in situ* during the time intervals between mid-October and mid-November 2015, the time interval during which rapid toluene increase was observed in the field data. While the actual mechanism may not be clear at this time, the results of the groundwater series without glucose but with added phenylalanine, seemed to more closely mimic the lag phase observed on site after molasses injection (Figure 3.1). *In situ* toluene production may result from microbial activity after the bulk of easily fermentable substrates had been consumed. Although sugars and fermentation products were not measured over time in groundwater or laboratory cultures reported here, previous experiments regarding molasses addition to groundwater from a different location at the same Superfund site (Narez, 2010) suggest that fermentation was rapid and free sugars did not persist beyond a few days after molasses addition. To further explore the impacts of exogenous carbon source supply on toluene accumulation, experiments were conducted using varying concentrations of glucose as described below in Section 3.4.3 and using alternative carbon sources as described below in Section 3.4.3.1.

### 3.4.3 Effect of Glucose Concentration on Toluene Production

Toluene-producing replicates from which utilized groundwater collected November 18, 2015 and further amended with only phenylalanine, as described above in Section 3.4.2 (GW +PA in Figure 3.4), were used as an inoculum in order determine the effects of varying glucose concentrations on toluene production. These replicates had been stored at 4° C for approximately 2 months after being analyzed. The culture was inoculated at 5% (vol./vol.) in Modified TP medium with varying concentrations of glucose and stored in the dark at ambient laboratory temperature (22°C). Glucose concentrations of 2,000 mg/L, 300 mg/L, and 0 mg/L glucose were established in 25 mL glass serum bottles with 15 mL liquid (medium and inoculum) and 10 mL gas head-space. All series contained phenylalanine at 132 mg/L and replicates analyzed at regular

intervals. Results of analysis are presented graphically in Figure 3.5. While toluene concentrations were nearly indistinguishable 7 days after inoculation, by Day 14 the resulting toluene accumulation was distinct for series supplied with different glucose concentrations. The series containing 2,000 mg/L glucose accumulated by far the most toluene, reaching its highest concentrations of  $40.1 \pm 1.7$  mg/L on Day 28. The series supplied with 300 mg/L glucose accumulated toluene to a maximum aqueous-phase concentration of  $12.04 \pm 0.01$  mg/L on Day 19, while the series not supplied with any glucose, accumulated toluene to a maximum of  $8.64 \pm 1.0$  mg/L on Day 32. Analysis on Day 35 of all negative controls, which consisted of un-inoculated Modified TP medium both with glucose (2000 mg/L) and without, had no measurable concentrations of toluene ( $<0.1$  mg/L), indicating that the toluene production was not abiotic. Attempts to further propagate these cultures in Modified TP medium were unsuccessful at accumulating appreciable levels of toluene (data not shown).



**Figure 3.5.** Toluene concentration as a function of time in time-series experiment utilizing Modified TP medium with “full-strength” glucose (2 g/L), reduced glucose (0.3 g/L), and no glucose.

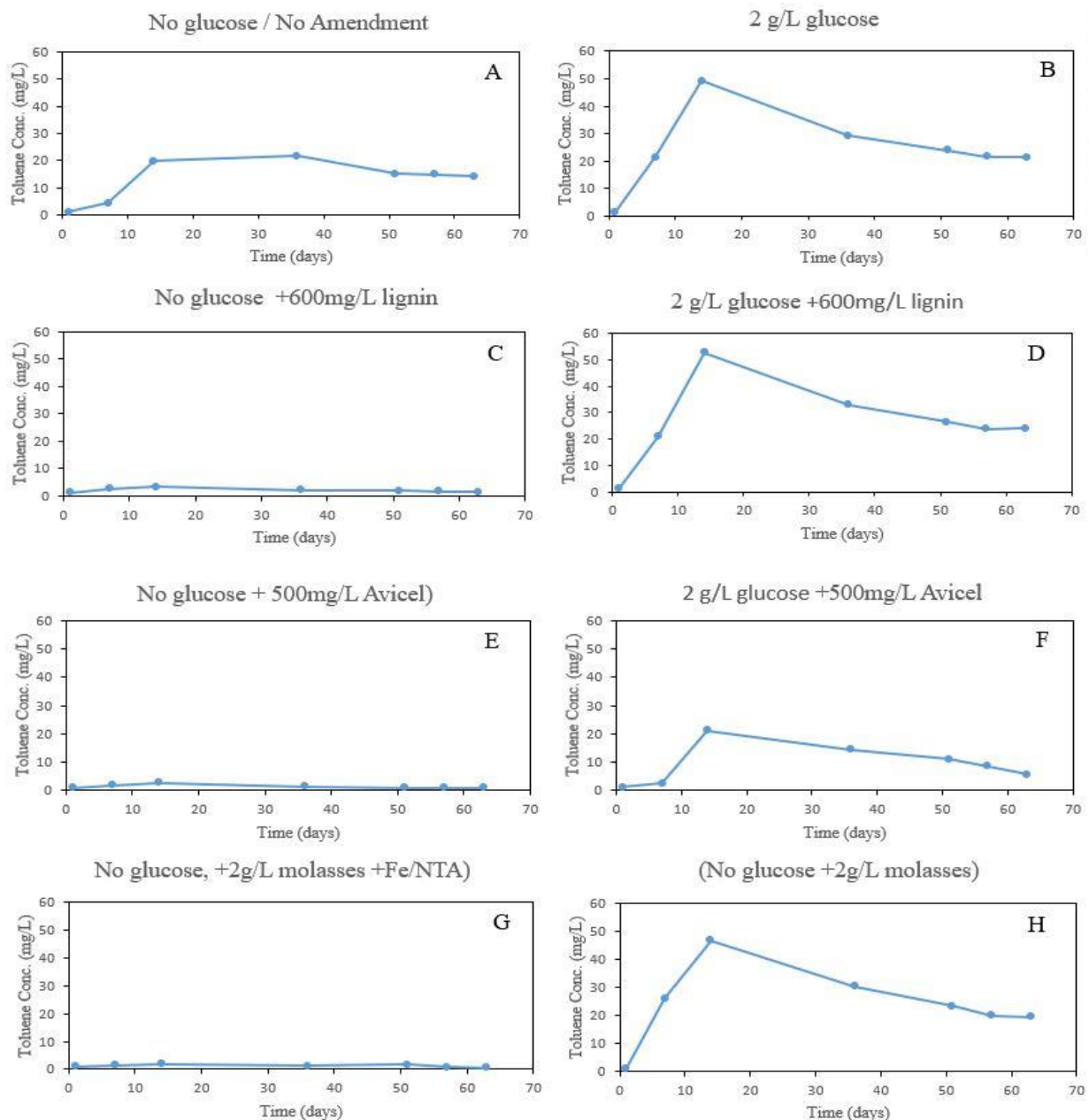


#### 3.4.4 Effect of Carbon Sources on Toluene Production

A time series experiment to study the effects of varying carbon sources on toluene production was established utilizing a groundwater-only microcosm, established from groundwater sampled on October 14, 2015 from Well SP024 (Figure 3.2), as the inoculum (5% volume/volume). The microcosm had been stored at 4° C for approximately 9 months prior to its use as an inoculum. Several series of various carbon sources were established, using the Modified TP Medium formulation as a basis. Series contained carbon sources Avicel or lignin in addition to glucose as well as Avicel or lignin instead of glucose. Two additional series utilized agricultural feed grade molasses (2 g/L) instead of glucose and agricultural feed grade molasses (2 g/L) with Iron(III) Nitritotriacetate (1 mM final concentration) instead of glucose.

Resulting analysis of toluene concentrations from each series are described below in Figure 3.6. Comparing series of Modified TP medium with 2g/L glucose (B) to that of medium with no glucose (A), toluene accumulation was again higher in the series with glucose than without. This is consistent with results shown previously (Figure 3.5) for a culture that came from the same well, approximately a month apart from each other. Comparing the results of series no glucose +lignin (C) and no glucose +Avicel (E) to that of no glucose (A) would seem to suggest that the presence of lignin and Avicel (cellulose) had some inhibitory effect on toluene production when no glucose was available. Also, while the series with lignin and glucose (D) had nearly indistinguishable effects on toluene accumulation to series with glucose only (B), the series with both glucose and Avicel (F) had noticeably less toluene accumulation. The series with agricultural feed grade molasses (H) exhibited toluene accumulation at a rate and extent quite similar observed for medium with glucose only (B), however the series with addition of Iron(III) Nitritotriacetate (G), however, exhibited dramatically lower toluene concentrations. Analysis of all negative controls,

which consisted of un-inoculated growth medium of the same formulation in each series, had no measurable concentration of toluene ( $<0.1$  mg/L), indicating that toluene production was biologically mediated. Attempts to propagate toluene-producing replicates utilizing the same formulations as their respective inoculum, as well as DSMZ Medium 500, were unsuccessful (data not shown).



**Figure 3.6.** Toluene concentration as a function of time in time-series experiment utilizing Modified TP medium formulation with varying carbon sources [A – H]

#### 3.4.5 Further Propagation Using October 2015 Microcosms and Effect of Varying Phenylalanine Concentration on Toluene Production

After multiple attempts to propagate toluene-producing cultures were unsuccessful (as noted in previous sub-sections), an experiment to study the effects of varying phenylalanine concentrations was established using the Modified TP Medium formulation. Final concentrations of phenylalanine were established at 0, 22, 44, 66, 88, 132, 220 and 440 mg/L. A replicate from the groundwater only microcosm experiment (Fig 3.2) established using groundwater collected from Well SP024 on 10/14/15 was used as the inoculum. After initial analysis (approximately 25 days after collection), the serum bottle used as inoculum had been stored at 4° C for approximately 15 months prior to use as inoculate. Inoculation was 5% (volume/volume) in 25 mL glass serum bottles containing 15 mL liquid medium. Bottles were analyzed for toluene after 42 days incubation at ambient laboratory temperature (22° C).

While this experiment did not provide consistent results (many replicates produced little or no toluene as shown in Appendix A1 Figure A.1), possibly due to low cell concentration after 15 months of storage, two replicates did produce a sizable concentration of toluene after the six-week incubation. One replicate supplied with 220 mg/L phenylalanine had accumulated 40.76 mg/L aqueous-phase toluene; another supplied 132 mg/L phenylalanine accumulated 31.57 mg/L aqueous-phase toluene.

Subsequently, 2 mL of the replicate which had accumulated 40.76 mg/L aqueous-phase toluene was used to inoculate two 160 mL serum bottles, each containing 100 mL of different growth medium. One formulation was based on the same medium as described by Zargar *et al.* (2016), (full protocol listed in Appendix A2: Zargar Medium). The other was the Modified TP medium, the variant based on the Fischer-Romero, (1996 formulation), which had been previously shown to produce toluene in first generation cultures but never in a serially-transferred generation.

For both medium formulations, phenylalanine was replaced with phenylacetic acid; both media had the same phenylacetic acid concentration, 27.2 mg/L.

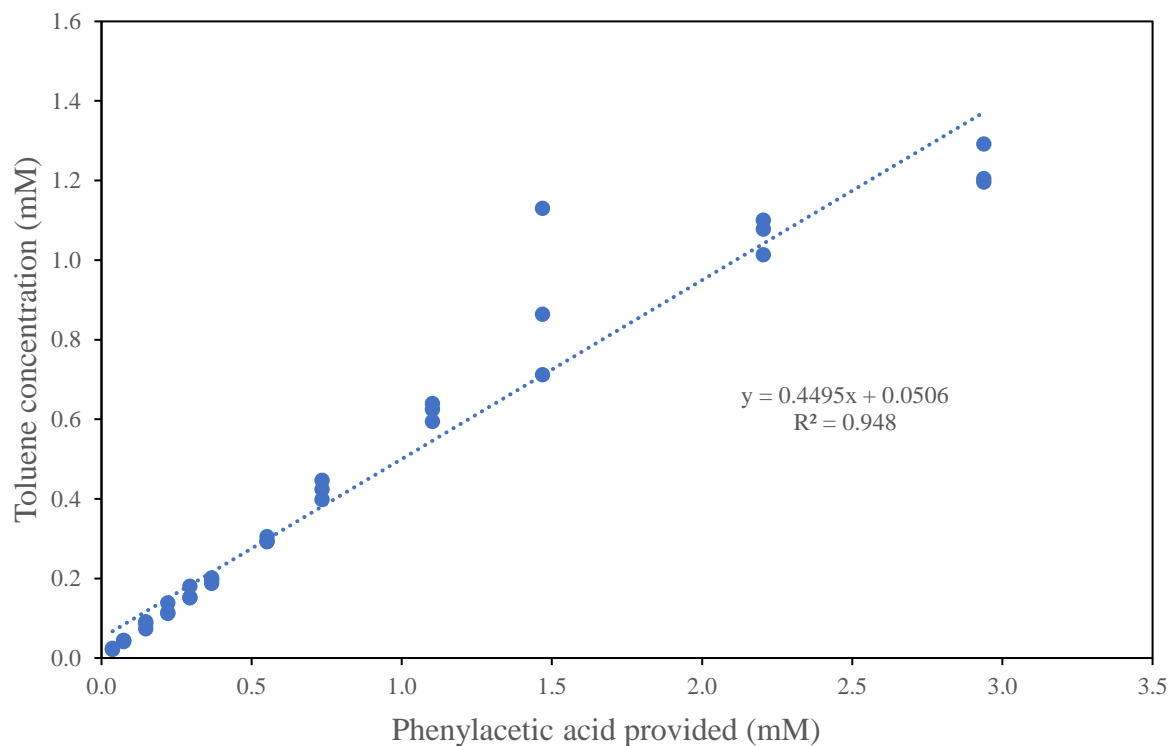
After 18 days incubation, the medium based on that of Zargar *et al.* (2016) had accumulated toluene to an aqueous-phase concentration of 19.4 mg/L. The replicate which utilized Modified TP medium based on the formulation of Fischer-Romero (1996) failed to accumulate aqueous phase toluene at detectable limits (<0.1 mg/L).

The production of toluene observed here was the first successful second-generation toluene-producing culture (considering the October 2015 microcosm shown in Figure 3.2 to be generation 0). The culture was again transferred into identical medium formulations, to that which it had come from, as described by Zargar *et al.* (2016). As previous, 2 mL of inoculum was used to inoculate three replicate 160 mL bottles, each containing 100 mL of growth medium (based on that of Zargar *et al.* (2016). After 28 days, analysis indicated that all three replicates had accumulated over 19 mg/L aqueous-phase toluene [third generation], providing a successful third generation propagation of the toluene producing culture. It is unknown why the TP medium based on that described by Fischer-Romero *et al.* (1996) was ineffective at sustaining toluene accumulation over multiple serial transfers. Nevertheless, the results demonstrate that the culture derived from Well SP024 could be successfully propagated in the medium based on that described by Zargar *et al.* (2016). The medium based on that described by Zargar *et al.* (2016), thus, was employed in subsequent experiments aimed at assessing the effects of varying phenyl-containing precursor concentrations on toluene accumulation.

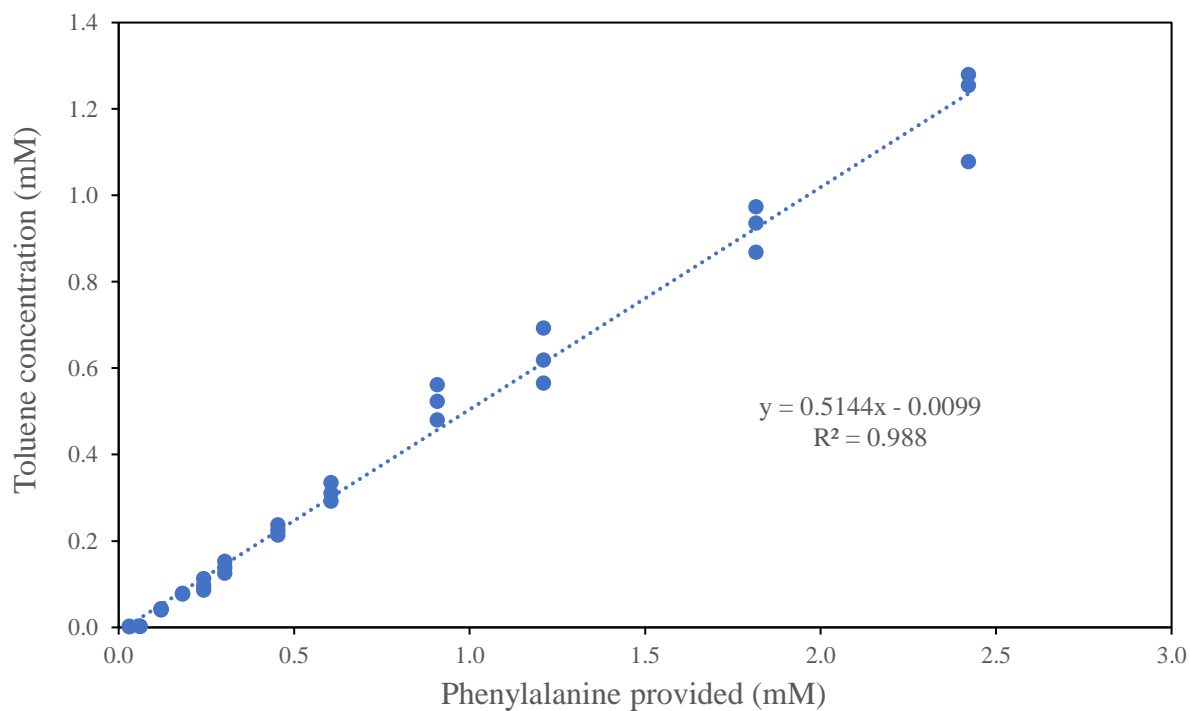
### 3.4.6 Varying Precursor Concentration Experimental Results

Utilizing a growth medium as described by Zargar *et al.* (2016), and another based on a similar formulation but replacing phenylacetic acid with phenylalanine, a varied precursor concentration experiment was established (see full details in Sec 3.3.4.4). Both formulations were inoculated with the second-generation enrichment culture, described above (Sec 3.4.5) which had a toluene concentration of 19.4 mg/L at the end of its incubation (prior to use as inoculum). Inoculation was 5% (volume/volume) in 25 mL serum bottles containing 15 mL medium and incubated at ambient laboratory temperature (22°C). Aqueous-phase toluene concentrations measured after 28 days incubation are shown in Figure 3.7 (for phenylacetic acid) and Figure 3.8 (for phenylalanine). At the highest concentration of phenylacetic acid supplied, 2.94 mM (400 mg/L), the final aqueous-phase concentration of toluene was  $1.23 \pm 0.05$  mM ( $113.4 \pm 4.9$  mg/L). At the highest concentration of phenylalanine supplied, 2.42 mM (400 mg/L), the final aqueous-phase concentration of toluene was  $1.28 \pm 0.11$  mM ( $117.9 \pm 10.1$  mg/L).

The total amount of toluene accumulated per bottle, shown in Figure 3.9 and Figure 3.10, was calculated assuming a Henry's Law dimensionless constant of 0.209; the experimentally measured aqueous-phase concentration and liquid and gas volumes per bottle. The sum of the aqueous-phase and gas phase concentrations multiplied by their respective volumes gave the total toluene produced (y-axis) which was plotted versus the total amount of precursor provided in the growth medium (x-axis). A linear regression was then fit to the data with correlation coefficients as shown in the figures.

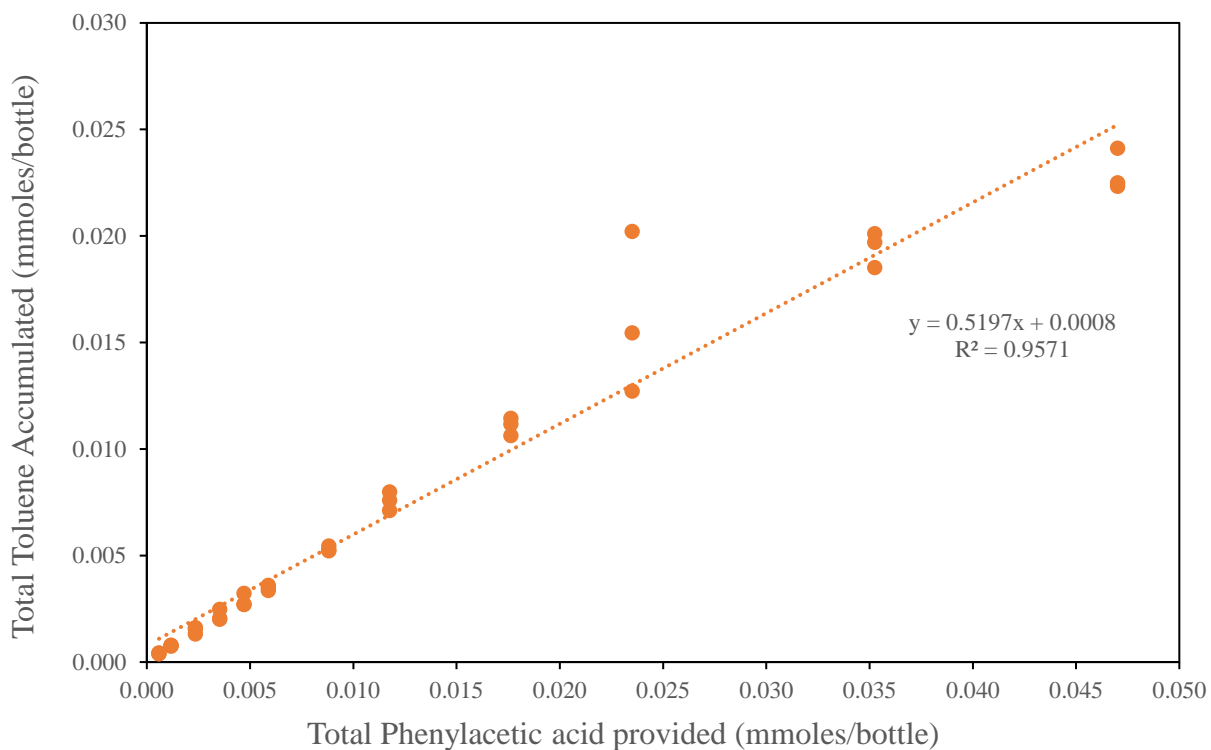


**Figure 3.7.** Aqueous-phase toluene concentration as a function of initial phenylacetic acid concentration provided in medium as described by Zargar *et al.* (2016) after 28 days incubation.

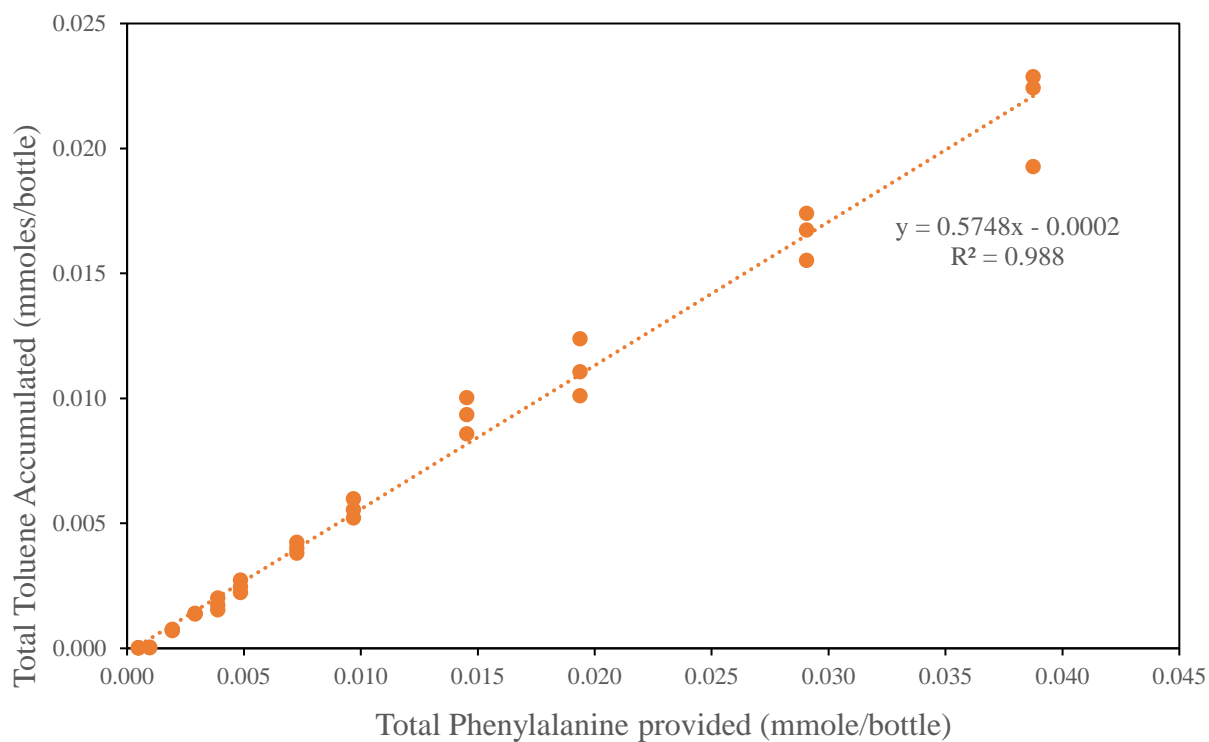


**Figure 3.8.** Aqueous-phase toluene concentration as a function of initial phenylalanine concentration provided in medium as described by Zargar *et al.* (2016) after 28 days incubation.

The molar ratio of toluene accumulated per phenylacetic acid provided was 0.52, lower than the yield reported by Zargar *et al.* (2016) for a wastewater-derived enrichment culture of 1.098 moles toluene produced per mole phenylacetic acid supplied. The lower toluene yield observed in the study reported here could be due to the potential sorption of some toluene to the butyl rubber stoppers used, as opposed to Zargar *et al.* (2016) experiments which utilized PTFE stoppers. Additionally, a portion of the phenylacetic acid precursor may have not been transformed by the culture resulting in a larger denominator term relative to the results reported by Zargar *et al.* (2016) who experimentally measured the amount of precursor consumed rather than calculating based on the amount of precursor initially provided. The molar ratio of toluene accumulated per phenylalanine supplied for the groundwater-derived enrichment culture reported herein was 0.575, somewhat higher than the 0.346 moles toluene accumulated per mole of phenylalanine consumed reported by Zargar *et al.* (2016). The apparently higher toluene yield from phenylalanine reported here may have been influenced by the culture being initially enriched using phenylalanine as a precursor. Regardless, there was a clear correlation between the supply of phenyl-containing precursor and the amount of toluene accumulation for both phenylacetic acid and phenylalanine.



**Figure 3.9.** Total toluene accumulated per bottle as a function of initial phenylacetic acid provided in medium as described by Zargar *et al.* (2016) after 28 days incubation.



**Figure 3.10.** Total toluene produced per bottle as a function of initial phenylalanine provided in medium as described by Zargar *et al.* (2016) after 28 days incubation.



### 3.4.7 Enrichment Cultures Established on Other Sampling Dates

Additional attempts to establish toluene-producing enrichment cultures utilizing groundwater collected at later dates as inoculum provided inconsistent results. However, groundwater collected from Well SP024 on July 18, 2016 showed a distinct potential for toluene production, seven months after the last notable *in situ* toluene concentration at this location. The groundwater obtained was transferred into Modified TP medium with phenylalanine (132 mg/L) as a precursor. While toluene concentrations were initially below 0.1 mg/L, two replicates analyzed on Day 30 showed aqueous-phase toluene concentrations of 42.8 mg/L and 45.5 mg/L; another replicate analyzed on Day 51 had an aqueous-phase toluene concentration of 25.5 mg/L. However, none of the other 21 replicates analyzed from this experimental series during the course of an incubation period lasting a total of 62 days, had detectable levels of toluene greater than 0.1 mg/L.

Groundwater collected from Well SP013 on November 21, 2016, also showed potential for toluene production, nine months after that last *in situ* toluene concentration above the 1 mg/L drinking water MCL at this location. The groundwater, which was transferred into Modified TP medium with phenylacetic acid (82 mg/L) as a precursor, had initial toluene concentrations below the detection limit (<0.1 mg/L). Of the duplicate serum bottles analyzed at 12 time-steps, over an incubation period lasting 21 days, a single replicate of the 24 bottles analyzed showed appreciable aqueous-phase toluene accumulation with 13.0 mg/L on Day 17. A plausible explanation for these inconsistent results could be a lack of viable cells present at high enough concentrations in the inoculum groundwater.

## 4 OVERALL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Experimental results described in this thesis provide compelling support for the notion that *in situ* microbial populations can biologically produce toluene in groundwater following subsurface injection of molasses. Furthermore, microbial populations able to produce toluene persisted in groundwater over time. Groundwater samples collected from Well SP024 in October 2015, November 2015, and July 2016 all yielded cultures that produced toluene under laboratory conditions. The toluene-production ability of the groundwater microbial population was able to persist in laboratory samples stored at 4°C for over 15 months. The toluene-producing microbial community was also found to be spatially distributed within the distal plume treatment zone with successful laboratory enrichment from groundwater collected from wells SP013 as well as SP024. Results of enrichment experiments provide supporting evidence that toluene producing cultures can be propagated utilizing medium as described by Zargar *et al.* (2016). Furthermore, with direct correlation of precursor supplied to toluene accumulated, there is strong evidence that both phenylalanine and phenylacetic acid can be converted into toluene.

Now that a viable medium has been identified, which appears capable of propagating a toluene-producing culture, there are multiple options available for future research. One recommendation for future research is to explore the pH range of which the cultures are capable of producing toluene. This may provide some insights, for those performing enhanced bioremediation treatment, on pH ranges which may avoid unintended toluene.

Further study could also be dedicated to on site monitoring of phenylalanine and phenyl acetate concentrations on site to distinguish which, if either, may be the underlying causes of *in situ* toluene production. In the same scope, as Fischer-Romero *et al.* (1996) also reported conversion of both phenyllactate and phenylpyruvate into toluene, in addition to the precursors

studied above, further study could be dedicated into other possible precursors which may be utilized by the enrichment cultures (or potential isolates).

Another recommendation for future research, would be the isolation and description of the microbial organism(s) responsible for toluene production. Because neither the TP medium as described by Fischer-Romero *et al.* (1996) nor the DSMZ 500 medium seemed to produce toluene, biogenically formed toluene at the site reported on in this thesis may be caused by a novel toluene-producing species. Identification of the microorganisms responsible for toluene production would facilitate development of techniques for monitoring their presence or abundance and could prove useful for ultimately identifying locations where enhanced bioremediation activities could unintentionally stimulation toluene production.

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## APPENDIX A: PROTOCOL FOR PREPARATION OF MEDIUM

### A1: General protocol for Modified TP medium

For initial toluene-production experiments, a modification of medium used for enrichment and isolation of *Tolomonas auensis* strains by Fischer-Romero *et al.* (1996) was employed. The medium as described by Fischer-Romero *et al.* (1996) was a modification of a medium (Eichler and Pfennig, 1988) that was a modification of a medium reported by Pfennig and Trüper (1981).

Using a clean 1 L orange-capped media bottle, add the following:

Constituent	Amount
Deionized water	936 mL
KH <sub>2</sub> PO <sub>4</sub>	250 mg
NH <sub>4</sub> Cl	340 mg
KCl	340 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	500 mg
CaCl·2H <sub>2</sub> O	250 mg
Resazurin solution (1 mg/mL) *	1 mL

\* Note: different from Fischer-Romero *et al.* (1996)

Tightly cap and then autoclave on liquids cycle (121°C and 15 psi, 15 min). Using a newly-sterilized needle and filter-sterilized gas supply, purge the liquid with filter sterilized N<sub>2</sub>/CO<sub>2</sub> gas. Once cool, add the following (recipes appear on following pages):

Constituent	Amount
Vitamin B <sub>12</sub> solution	1 mL
Trace element solution SL10	1 mL
NaHCO <sub>3</sub> solution	10 mL
Glucose solution	20 mL
Yeast extract solution	2 mL
Phenylalanine solution	20 mL

The constituents listed in the table above can be added to the bottle of cooled medium inside an anaerobic chamber. After adding the constituents, use a sterile sampling device (sterile pipette or sterile syringe) to transfer a small aliquot (~5 mL) to a small container and measure pH. Adjust to pH 7.2 using 2 M sterile stock solution of anoxic HCl or NaOH.

### Vitamin B<sub>12</sub> solution

In a clean glass container, add 2 mg vitamin B<sub>12</sub> to 100 mL distilled water. Filter sterilize, transferring to a sterile serum bottle. Seal with sterile butyl rubber stopper and aluminum crimp cap and then purge headspace gas with filter-sterilized N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub> for 5 minutes.

### Trace Element Solution SL10

Using a clean 1 L orange-capped media bottle, add the following:

Constituent	Amount
Deionized water	500 mL
HCl (37%)	4.25 mL
FeCl <sub>2</sub> ·4H <sub>2</sub> O	0.75 g
H <sub>3</sub> BO <sub>3</sub>	3 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	50 mg
CoCl <sub>2</sub> ·6H <sub>2</sub> O	95 mg
Zinc Acetate ·2H <sub>2</sub> O*	72 mg
NiCl <sub>2</sub> ·6H <sub>2</sub> O	12 mg
CuCl <sub>2</sub> ·2H <sub>2</sub> O	1 mg
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	18 mg

\*Note: different from Fischer-Romero *et al.* (1996)

Cap bottle and shake to dissolve all constituents. Then, divide the resulting 500 mL of solution evenly between two sterile 500 mL serum bottles (i.e., 250 mL each). Cap with aluminum crimp caps and butyl rubber stoppers. Purge gas headspace with N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub>, and then autoclave.

### NaHCO<sub>3</sub> solution

NaHCO<sub>3</sub>            15 g  
Water                fill to 100 mL

Dissolve 15 g NaHCO<sub>3</sub> into 100 mL total volume using distilled water in a 162 mL serum bottle. Seal the bottle with rubber stopper and aluminum cap, and purge the headspace with N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub> gas for 5 min. Autoclave the bottle on liquid cycle (121°C) for 15 min.

### Glucose solution

Glucose    20 g  
Water        fill to 200 mL

Dissolve 20 g glucose in a clean 500-mL beaker along with deionized water to reach a final liquid volume of 200 mL. Filter sterilize solution into autoclaved serum bottles on clean bench using aseptic techniques. Cap with sterile butyl rubber stopper and aluminum crimp cap, and then purge the gas headspace with filter sterilized N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub> gas for 5 min.

### Yeast extract solution

Yeast extract    0.5 g  
Water             50 mL

Place 0.5 g yeast extract in a 100-mL serum bottle along with 50 mL deionized water. Cap with butyl rubber stopper and aluminum crimp cap, and then purge the headspace with N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub> gas for 5 min. Autoclave the bottle on liquid cycle (121°C) for 15 min.

### Phenylalanine solution

Phenylalanine	660 mg
Water	100 mL

Place 660 mg phenylalanine and 100 mL water in a 162-mL serum. Filter sterilize solution into autoclaved serum bottles on clean bench using aseptic techniques. Cap with butyl rubber stopper and aluminum crimp cap, and then purge the headspace with N<sub>2</sub> or N<sub>2</sub>/CO<sub>2</sub> gas for 5 min.

### A2: General protocol for Zargar Medium Protocol for Preparation of “Zargar (2016) medium”

Boil water for 20 minutes. While water is heating and boiling, add the constituents listed below to a clean 500 mL glass serum bottle. Carefully pour 350 mL boiled water into the serum bottle, and then cap with red butyl rubber stopper. Insert a hypodermic needle through the butyl stopper (to serve as a gas vent), and then insert a long (6”) stainless steel needle connected to an O<sub>2</sub>-free gas cylinder (95% N<sub>2</sub>: 5% CO<sub>2</sub>) through the butyl stopper and until it reaches near bottom of the liquid. Purge with anaerobic gas 5 minutes. Withdraw the vent needle, and then 5 seconds later withdraw the gas supply syringe. Autoclave to sterilize (use liquids cycle, 121°C, 15 min, 1.43 atm).

The list of constituents for Zargar (2016) medium and the quantity of those constituents per 0.35L

Constituent	Quantity per 0.35 L
KH <sub>2</sub> PO <sub>4</sub>	87.5 mg
NH <sub>4</sub> Cl	119 mg
KCl	119 mg
Sodium HEPES	1,640 mg
Glucose	350 mg
MgCl <sub>2</sub> ·6H <sub>2</sub> O	350 mg
MgSO <sub>4</sub> ·7H <sub>2</sub> O	35 mg
CaCl <sub>2</sub> ·2H <sub>2</sub> O	43.8 mg
Phenylacetic acid stock solution	3.5 mL
Yeast extract stock solution (see attached)	3.5 mL
Trace element solution (see attached)	0.175 mL
1M HCl solution*	2.5 mL*

\*Note: after adding all of the above constituents to 350 mL boiled water, purging, and autoclaving, the pH of solution is about 7.9 IF the 1 M HCl solution is omitted. Note that there will be some particulate material visible in the bottles prior to pH adjustment. Most or all of the visible particulate material will dissolve when the pH is adjusted from 7.9 to 7.1.

\*\*For varying concentration experiment, phenylacetic acid solutions of varying concentrations will be added (all at 1.0 mL volumes) after media has been dispense, but prior to inoculation.

After the bottles are finished autoclaving, cool to room temperature. Remove an aliquot and check the pH (it should be ~7.1). If not at pH 7.1 after autoclaving, the pH should be adjusted to pH 7.1 by adding a filter-sterilized 1 M HCl or 1 M NaOH solution using a sterile disposable syringe and needle.

Transfer into the anaerobic chamber. Once inside the anaerobic chamber, use a 1 mL sterile syringe and hypodermic needle to add 0.1 mL vitamin B12 stock solution (see attached) per 350 mL medium.

Using aseptic and strictly anaerobic technique, dispense into sterile serum bottles and seal with sterile butyl rubber stoppers and crimp caps.

### **Protocols for Preparation of Stock solutions for use in preparing “Zargar medium”**

#### **Phenylacetic acid stock solution (100×)**

Add 272 mg phenylacetic acid (Aldrich, P16621-100G) to a clean 162 mL glass serum bottle. Boil water for 20 minutes. Carefully pour 100 mL boiled water into the serum bottle containing the phenylacetic acid. Cap with a butyl rubber stopper and aluminum crimp cap. Working underneath a functioning snorkel to exhaust lab air, insert a hypodermic needle through the butyl stopper (to serve as a gas vent), and then insert a long (6”) stainless steel needle connected to an O<sub>2</sub>-free gas cylinder (95% N<sub>2</sub>: 5% CO<sub>2</sub>) through the butyl stopper and until it reaches the bottom of the liquid. Purge with anaerobic gas 5 minutes. Withdraw the vent needle, and then 5 seconds later withdraw the gas supply syringe. Autoclave to sterilize.

\*\* For varying precursor concentration experiment, stock solutions of both phenylacetic acid and phenylalanine were established at 3000, 2250, 1500, 1125, 750, 600, 450, 300, 150, and 75 mg/L were used.

#### **Yeast extract stock solution (100×)**

Add 100 mg yeast extract (Bacto Yeast Extract, BD) to a clean 162 mL glass serum bottle. Boil water for 20 minutes. Carefully pour 100 mL boiled water into the serum bottle containing yeast extract. Cap with a grey butyl rubber stopper and aluminum crimp cap. Insert a hypodermic needle through the butyl stopper (to serve as a gas vent), and then insert a long (6”) stainless steel needle connected to an O<sub>2</sub>-free gas cylinder (95% N<sub>2</sub>: 5% CO<sub>2</sub>) through the butyl stopper and until it reaches the bottom of the liquid. Purge with anaerobic gas 5 minutes. Withdraw the vent needle, and then 5 seconds later withdraw the gas supply syringe. Autoclave to sterilize.

**Trace element solution (2000×**

Prepare the trace element solution by Zargar *et al.*, (2016) by dissolving the following constituents in 100 mL of deionized water in a 162-mL glass serum bottle. Seal with grey butyl rubber stopper and aluminum crimp cap. Insert a disposable needle connected to an O<sub>2</sub>-free gas cylinder (95% N<sub>2</sub>: 5% CO<sub>2</sub>) through the butyl stopper into the gas headspace and insert a separate vent hypodermic needle through the butyl stopper to serve as a vent. Purge the gas headspace with anaerobic gas 5 minutes. Withdraw the vent needle, and then 5 seconds later withdraw the gas supply syringe. Autoclave to sterilize.

Constituent	Quantity per 100 mL
7.7 N (25%) HCl*	1.25 mL
FeSO <sub>4</sub> ·7H <sub>2</sub> O	210 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	10 mg
CoCl <sub>2</sub> ·6H <sub>2</sub> O	19 mg
ZnCl <sub>2</sub>	7 mg
NiCl <sub>2</sub> **	1.3 mg
CuCl <sub>2</sub> ·2H <sub>2</sub> O	0.2 mg
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	3.6 mg
H <sub>3</sub> BO <sub>3</sub>	0.6 mg

\* In making trace element solution on 3/16/17, used 37% HCl and changed volume to 0.83 mL to get same concentration in final solution using the acid that we had in lab

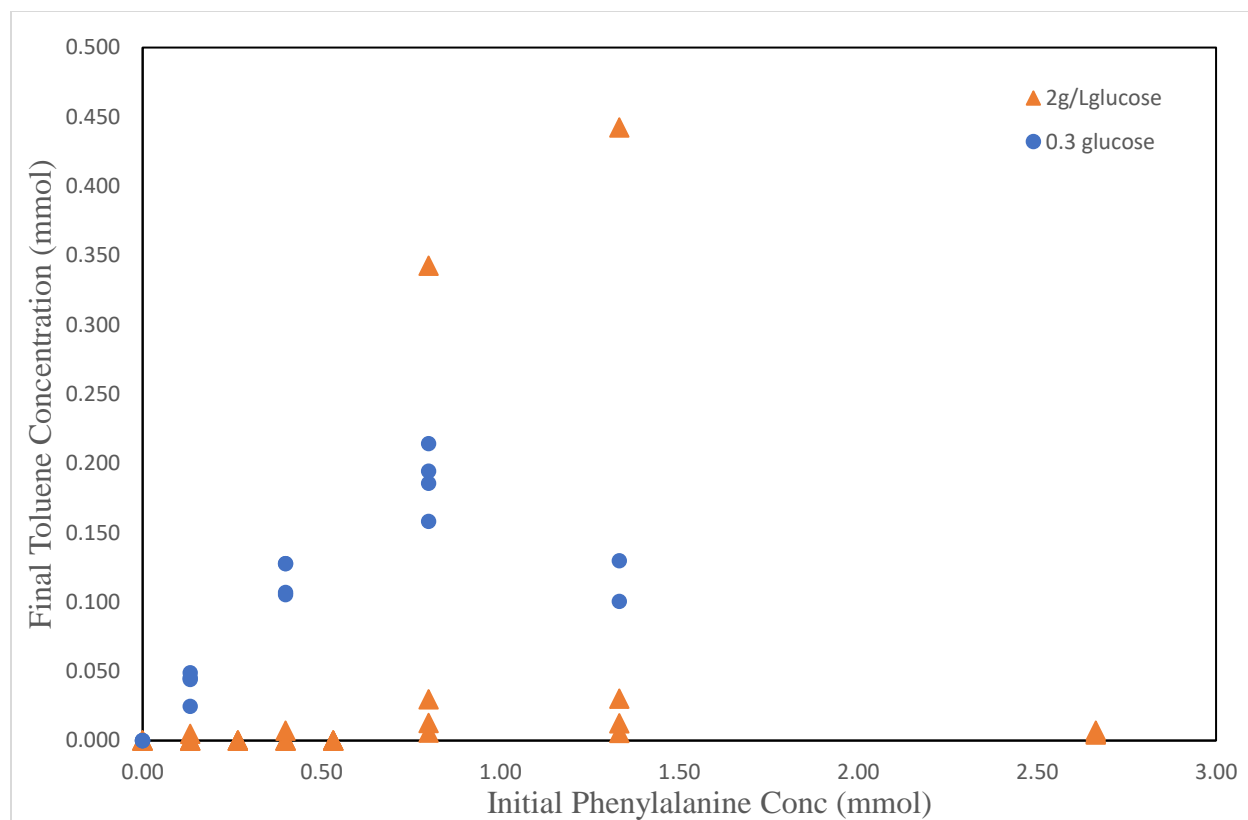
\*\* added in the form of NiCl<sub>2</sub>·6H<sub>2</sub>O, so added 2.4 mg to match molar concentration

**Vitamin B12 stock solution (5000×**

Add 10 mg vitamin B12 (Sigma-Aldrich cat. # V2876-100mg) to a clean beaker. Add 100 mL deionized water. Stir with a pipette to dissolve. Filter sterilize (using a Fisher Brand 0.2 µm sterile nylon filter, cat # 09-719C) and transfer to sterile 162 mL glass serum bottle. Cap with a sterile grey butyl rubber stopper and aluminum crimp cap. Wrap the bottle in aluminum foil or opaque tape. Using a filter-sterilized gas supply, purge the gas headspace with O<sub>2</sub>-free gas for 5 minutes. Withdraw the vent needle, and then 5 seconds later withdraw the gas supply syringe. Store in refrigerator.

**Table A2** Side by side comparison of relevant medium formulations

	<i>Tolomonas</i> isolation (Fischer- Romero <i>et al.</i> , 1996)	Modified TP Medium	Zargar <i>et al.</i> (2016) enrichment medium
KH <sub>2</sub> PO <sub>4</sub>	250 mg/L	250 mg/L	250 mg/L
NH <sub>4</sub> Cl	340 mg/L	340 mg/L	340 mg/L
KCl	340 mg/L	340 mg/L	340 mg/L
MgSO <sub>4</sub> * 7H <sub>2</sub> O	500 mg/L	500 mg/L	100 mg/L
CaCl <sub>2</sub> * 2H <sub>2</sub> O	250 mg/L	250 mg/L	125 mg/L
MgCl <sub>2</sub> ·6H <sub>2</sub> O	---	---	1000 mg/L
FeCl <sub>2</sub> * 4H <sub>2</sub> O	0.75 mg/L	0.75 g/L	---
H <sub>3</sub> BO <sub>3</sub>	0.003 mg/L	0.003 mg/L	0.003 mg/L
MnCl <sub>2</sub> * 4H <sub>2</sub> O	0.05 mg/L	0.05 mg/L	0.05 mg/L
CoCl <sub>2</sub> * 6H <sub>2</sub> O	0.095 mg/L	0.095 mg/L	0.095 mg/L
ZnCl <sub>2</sub>	0.035 mg/L	---	0.035 mg/L
Zinc Acetate	---	0.047 mg/L	---
NiCl <sub>2</sub> * 6H <sub>2</sub> O	0.012 mg/L	0.012 mg/L	0.012 mg/L
CuCl <sub>2</sub> * 2H <sub>2</sub> O	0.001 mg/L	0.001 mg/L	0.001 mg/L
Na <sub>2</sub> MoO <sub>4</sub> * 2H <sub>2</sub> O	0.018 mg/L	0.018 mg/L	0.018 mg/L
FeSO <sub>4</sub> ·7H <sub>2</sub> O	---	---	1.05 mg/L
NaHCO <sub>3</sub>	1.5 g/L	1.5 g/L	---
Sodium HEPES	---	---	4.685 g/L
Na <sub>2</sub> S	5 mmol Na <sub>2</sub> S	---	---
C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> (Glucose)	1 g/L	2 g/L	1 g/L
yeast extract	10 mg/L	10 mg/L	10 mg/L
Vitamin B <sub>12</sub>	.02 mg/L	.02 mg/L	.03 mg/L
Adjusted pH [using sterile HCl or NaOH (2M each)]	7.2	7.2	7.1



**Figure A.1.** Toluene concentration per bottle as a function of initial phenylalanine concentration after 6 weeks of incubation.



## APPENDIX B: GROUNDWATER CONTAMINANT AND GEOCHEMISTRY DATA

**Table B1.** Contaminant concentrations measured in groundwater collected from well SP013 (data from NPC Services, Inc).

Analyte*	11/21/16
1,1-Dichloroethane (µg/L)	<0.578 <sup>J</sup>
1,1-Dichloroethene (µg/L)	1.23
1,2-Dichloroethane (µg/L)	19.3
1,2-Dichloropropane (µg/L)	60.0
1,3-Dichlorobenzene (µg/L)	0.355 <sup>J</sup>
1,4-Dichlorobenzene (µg/L)	0.580 <sup>J</sup>
Benzene (µg/L)	0.642 <sup>J</sup>
Chlorobenzene	1.44
Chloroethane	2.32
<i>cis</i> -1,2-Dichloroethene (µg/L)	73.6
Ethylbenzene (µg/L)	<1 <sup>a</sup>
Methylene Chloride (µg/L)	1.25
1,1,2,2-Tetrachloroethane (µg/L)	<1 <sup>a</sup>
Tetrachloroethene (µg/L)	3.59
Toluene (µg/L)	11.4
<i>trans</i> -1,2-Dichloroethene (µg/L)	3.1
1,1,2-Trichloroethane (µg/L)	<1 <sup>a</sup>
Trichloroethene (µg/L)	8.82
Vinyl chloride (µg/L)	112

\*Analyzed using US EPA Method 8260B

<sup>J</sup> Estimated value (result between the method detection limit and limit of quantitation)

<sup>a</sup> Denotes method detection limit, therefore maximum amount possible

**Table B2.** Geochemical parameters measured in groundwater collected from well SP013 (data from NPC Services, Inc).

Analyte*	11/21/16
Ethane (µg/L)	0.293 <sup>J</sup>
Ethene (µg/L)	3.41
Methane (µg/L)	13700
Nitrate (mg/L-N)	<0.01 <sup>a</sup>
Nitrite (mg/L-N)	0.02
Sulfate (mg/L)	2.39
Total Alkalinity (mg/L as CaCO <sub>3</sub> )	1770
Ferrous iron (mg/L)	16.6
Chloride (mg/L)	6760
Sulfide (mg/L)	3.21
Total organic carbon (mg/L)	974

\*Analyzed as described by Bowman *et al.* (2006)

<sup>J</sup> Estimated value (result between the method detection limit and limit of quantitation)

<sup>a</sup> Denotes upper detection limit, therefore maximum amount possible

**Table B3.** Contaminant concentrations measured in groundwater collected from well SP024 (data from NPC Services, Inc).

Analyte*	5/22/13	6/13/13	9/25/13	2/4/14	4/23/14	7/14/14	2/26/15	8/6/15	10/14/15	11/3/15	11/18/15
1,1-Dichloroethane (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
1,1-Dichloroethene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
1,2-Dichloroethane (µg/L)	2.42	3.24	3	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	9.11	5.36	<40 <sup>a</sup>	<4 <sup>a</sup>
1,2-Dichloropropane (µg/L)	2.12	5.47	2.9	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	0.659	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Benzene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	0.264 <sup>J</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Chlorobenzene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
<i>cis</i> -1,2-Dichloroethene (µg/L)	<1 <sup>a</sup>	0.54 <sup>J</sup>	8.45	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Ethylbenzene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Methylene Chloride (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
1,1,2,2-Tetrachloroethane (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Tetrachloroethene (µg/L)	<1 <sup>a</sup>	**	**	**	**	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	0.926 <sup>J</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Toluene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	139	29000	15000	4800	1.19	1.77	103	19000	2,790
<i>trans</i> -1,2-Dichloroethene (µg/L)	<1 <sup>a</sup>	<1 <sup>a</sup>	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
1,1,2-Trichloroethane (µg/L)	8.87	14.3	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	<1 <sup>a</sup>	0.951	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Trichloroethene (µg/L)	<1 <sup>a</sup>	**	**	**	**	<50 <sup>a</sup>	<1 <sup>a</sup>	<5 <sup>a</sup>	0.718 <sup>J</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>
Vinyl chloride (µg/L)	<1 <sup>a</sup>	1.33	<1 <sup>a</sup>	<500 <sup>a</sup>	<200 <sup>a</sup>	<50 <sup>a</sup>	1.03	<5 <sup>a</sup>	<0.2 <sup>a</sup>	<40 <sup>a</sup>	<4 <sup>a</sup>

\*Analyzed using US EPA Method 8260B

\*\*data not available

<sup>J</sup> Estimated value (result between the method detection limit and limit of quantitation)<sup>a</sup> Denotes upper detection limit, therefore maximum amount possible

(Table B3. Continued)

Analyte*	12/16/15	3/3/16	7/18/16
1,1-Dichloroethane (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
1,1-Dichloroethene (µg/L)	<20 <sup>a</sup>	<0.2	<0.2 <sup>a</sup>
1,2-Dichloroethane (µg/L)	<20 <sup>a</sup>	0.416 <sup>J</sup>	<0.2 <sup>a</sup>
1,2-Dichloropropane (µg/L)	<20 <sup>a</sup>	0.504 <sup>J</sup>	0.689 <sup>J</sup>
Benzene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Chlorobenzene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
<i>cis</i> -1,2-Dichloroethene (µg/L)	<20 <sup>a</sup>	0.557 <sup>J</sup>	<0.2 <sup>a</sup>
Ethylbenzene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Methylene Chloride (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
1,1,2,2-Tetrachloroethane (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Tetrachloroethene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Toluene (µg/L)	14,900	146	2.71
<i>trans</i> -1,2-Dichloroethene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
1,1,2-Trichloroethane (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Trichloroethene (µg/L)	<20 <sup>a</sup>	<0.2 <sup>a</sup>	<0.2 <sup>a</sup>
Vinyl chloride (µg/L)	<20 <sup>a</sup>	0.821	<0.2 <sup>a</sup>

\*Analyzed using US EPA Method 8260B

<sup>J</sup> Estimated value (result between the method detection limit and limit of quantitation)

<sup>a</sup> Denotes upper detection limit, therefore maximum amount possible

**Table B4.** Geochemical parameters measured in groundwater collected from well SP024 (data from NPC Services, Inc).

Analyte*	5/22/13	6/13/13	9/25/13	2/4/14	4/23/14	7/2/14	2/26/15	8/6/15	10/14/15	11/3/15	11/18/15
Ethane (µg/L)	**	**	<1.0 <sup>a</sup>	<1.0 <sup>a</sup>	<1.0 <sup>a</sup>	<1.0 <sup>a</sup>	1.57	**	<0.11 <sup>a</sup>	0.3	0.199 <sup>J</sup>
Ethene (µg/L)	**	**	0.487 <sup>J</sup>	<1.0 <sup>a</sup>	<1.0 <sup>a</sup>	1.1	0.931	**	0.637 <sup>J</sup>	1.99	2.67
Methane (µg/L)	**	**	21200	9640	24700	11500	9710	**	14,100	17,700	13,700
Nitrate (mg/L-N)	**	**	<0.01 <sup>a</sup>	<0.01 <sup>a</sup>	<0.01 <sup>a</sup>	0.03	<0.01 <sup>a</sup>	0.154	<0.008 <sup>a</sup>	<0.008 <sup>a</sup>	<0.008 <sup>a</sup>
Nitrite (mg/L-N)	**	**	0.053	0.052	<0.01 <sup>a</sup>	0.026	0.097	**	0.018	0.027	0.085
Sulfate (mg/L)	**	**	0.956 <sup>J</sup>	<1.0 <sup>a</sup>	1.85	4.34	<0.2 <sup>a</sup>	81.1	4.95	<1.0 <sup>a</sup>	0.798
Total Alkalinity (mg/L as CaCO <sub>3</sub> )	**	1700	3980	3220	2180	1890	2430	**	1,880	2,030	3,910
Ferrous iron (mg/L)	**	**	7.93	<0.25 <sup>a</sup>	1.89	1.65	3.12	**	10.5	3.87	n/a
Chloride (mg/L)	**	**	89.9	59.1	69.4	48.1	68.7	105	50.4	51.2	52.9
Sulfide (mg/L)	**	**	0.077	0.027	0.098	0.107	0.1	**	2.89	2.07	1.75
Total organic carbon (mg/L)	**	1730	1870	322	215	**	204	1930	1,420	1,500	992
pH (SU)	**	**	7.2	7.2	7.4	7.67	7.52	**	6.48	**	6.75
ORP (mV)	**	**	-347	-243	-263	-288	-305	**	-341	**	-240
Temperature (°C)	**	**	20.2	19.4	19.8	20.23	18.6	**	20.9	**	20
Conductivity (µS)	**	**	7651	5532	4404	3541	4251	**	4,351	**	4710
Turbidity (NTU)	**	**	**	**	**	28.36	**	**	83 <sup>b</sup>	**	45.45

\*Analyzed as described by Bowman *et al.* (2006)

\*\*data not available

<sup>J</sup>Estimated value (result between the method detection limit and limit of quantitation)<sup>a</sup>Denotes upper detection limit, therefore maximum amount possible

(Table B4. Continued)

Analyte*	12/16/15	3/3/16	7/18/16
Ethane (µg/L)	<0.11 <sup>a</sup>	3.3	7.53
Ethene (µg/L)	2.59	3.01	<0.15 <sup>a</sup>
Methane (µg/L)	10,400	14300	23000
Nitrate (mg/L-N)	<0.008 <sup>a</sup>	<0.008 <sup>a</sup>	<0.008 <sup>a</sup>
Nitrite (mg/L-N)	0.026	0.017	0.022
Sulfate (mg/L)	1.22	2.34	1.24 <sup>J</sup>
Total Alkalinity (mg/L as CaCO <sub>3</sub> )	2,800	2370	1430
Ferrous iron (mg/L)	4.39	3.25	2.58
Chloride (mg/L)	64.7	66.3	52.2
Sulfide (mg/L)	0.029	1.87	<0.098 <sup>a</sup>
Total organic carbon (mg/L)	123	102	88.3
pH (SU)	7.15	7.32	7.04
ORP (mV)	-234	-255	-211
Temperature (°C)	20	20.2	20.4
Conductivity (µS)	4731	4519	2906
Turbidity (NTU)	30.57	30.6	10.7

\*Analyzed as described by Bowman *et al.* (2006)

\*\*data not available

<sup>J</sup> Estimated value (result between the method detection limit and limit of quantitation)

<sup>a</sup> Denotes upper detection limit, therefore maximum amount possible

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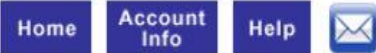
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**Author:** E A Edwards, D Grbić-Galić

**Publication:** Applied and Environmental Microbiology

**Publisher:** American Society for Microbiology

**Date:** Jan 1, 1994

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**Title:** Anaerobic Mineralization of  
Toluene by Enriched Sediments  
with Quinones and Humus as  
Terminal Electron Acceptors

**Author:** Francisco J. Cervantes, Wouter  
Dijksma, Tuan Duong-Dac et al.

**Publication:** Applied and Environmental  
Microbiology

**Publisher:** American Society for  
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**Date:** Oct 1, 2001

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**Publication:** Nature

**Publisher:** Nature Publishing Group

**Date:** Oct 30, 1986

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**Author:** Anne Cornish Frazer, Peter W. Coschigano, Lily Y. Young

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**Publication:** Anaerobe  
**Publisher:** Elsevier  
**Date:** December 1995

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**Title:** Succinyl-CoA:(R)-  
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Anaerobic Toluene Catabolic  
Pathway in Denitrifying Bacteria

**Author:** Christina Leutwein, Johann  
Heider

**Publication:** Journal of Bacteriology

**Publisher:** American Society for  
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**Date:** Jul 15, 2001

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## **VITA**

M. Aaron Griffin Jr., first born of Michael Griffin and Jerrienne Landaiche, was raised in St. Gabriel, La. Aaron spent a substantial portion of his youth attending a multitude of LSU sporting events with the most dedicated LSU sports fan, his grandmother Dora Landaiche. After graduating from East Ascension High School, Aaron moved to Atlanta, Ga. where he worked as a Portability Specialist for the H.U.D. Section 8 - Rental Assistance Program administered by the State of Georgia. After learning of LSU's bachelor's in environmental engineering program, Aaron returned to Louisiana and began attending LSU in Spring of 2012. After completing his Bachelor of Science in Environmental Engineering in May 2015, Aaron was inspired to apply to graduate school by his professor and mentor Dr. William Moe, with whom as an undergrad, he was employed as a lab assistant. Aaron is a candidate to receive his Master's of Science degree in Civil Engineering and plans to collect his diploma during the May 2018 commencement ceremony.